A Genetic and Molecular Study of Pollen Tube Growth in *Arabidopsis*

Een wetenschappelijke proeve op het gebied van Natuurwetenschappen,

Wiskunde en Informatica

Proefschrift

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Contents

Chapter 1

General Introduction

Sexual plant reproduction

The life cycle of flowering plants is characterized by an alternation of the sporophytic generation and the haploid generation formed by the male and female gametophytes within the flower (figure 1). In most angiosperms, the female gametophyte or embryo sac consists of seven cells: three antipodal cells at the chalazal end, a large central cell containing two polar nuclei, and two synergid cells and the egg cell at the micropylar end. The mature male gametophyte or pollen is composed of two sperm cells enclosed within one vegetative cell. Upon contact with the stigma, the vegetative cell undergoes dramatic polarized growth and coordinates the delivery of the two male gametes to the embryo sac for double fertilization. One sperm cell fuses with the egg cell, forming the diploid zygote, and the other with the central cell, resulting in triploid endosperm that nourishes the embryo through seed development (reviewed in Weterings and Russell, 2004). Here, we focus on the development and function of the male gametophyte leading to fertilization, and in particular on genes expressed in pollen that are involved in these processes. During the last decade, most genetic and molecular studies used *Arabidopsis thaliana* as model*,* as this plant of the Brassicaceae family has the advantage of a short generation time, a fully sequenced genome and the availability of mutant populations that correspond to a mutation in almost every gene (*Arabidopsis* Genome Initiative, 2000; Alonso *et al.*, 2003). Mutants in sexual reproduction are either sporophytic or gametophytic mutants, depending on the expression pattern of the mutated genes. Sporophytic mutants have a mutation in genes expressed in sporophytic tissues and these mutations are generally recessive. Homozygous mutations in sporophytic genes involved in pollen development or pollen tube growth generally lead to a defect in all pollen grains (reviewed in Scott *et al.,* 2004). Gametophytic mutants however, have a mutation in genes that are expressed by the pollen itself. In heterozygous gametophytic mutants, only half of the pollen grains contains the mutated allele and will be affected, while the other half carries the wild-type allele and thus will function normally (Howden *et al.,* 1998).

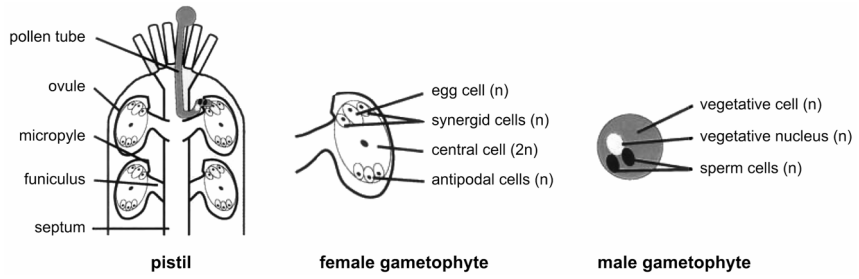

Figure 1: The male and female gametophytes in flowering plants
(adjusted from Johnson *et al.*, 2004)

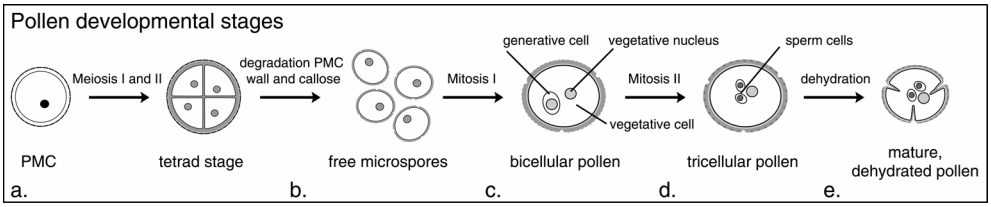

Figure 2: Pollen development

Schematic representation of the different developmental stages of pollen development. a. meiotic division of the pollen mother cell (PMC) resulting in four microspores tetrahedrically attached to each other, b. degradation of pollen mother cell wall and callose by enzymes derived from the tapetum, c. asymmetric mitotic division, d. second mitotic division of the generative cell resulting in two sperm cells, e. pollen dehydration.

Pollen development

Pollen development in *Arabidopsis* follows the general pattern of pollen development seen in most angiosperms (Owen and Makaroff, 1995), and mutants of *Arabidopsis* affected at any stage of pollen development or pollen tube growth have been identified (reviewed in Twell, 2002; McCormick, 2004). Pollen development starts with the meiotic division of the diploid microspore mother cell, resulting in four haploid microspores (figure 2a). The microspores are positioned in tetrads within a thick callose wall and surrounded by the remaining wall of the microspore mother cell. This tetrad stage is ended by the degradation of the microspore mother cell wall and the callose wall by enzymes from the tapetum, releasing the single microspores (figure 2b). The *Arabidopsis* sporophytic *quartet* mutants, each mutated in a gene coding for one of these enzymes, show a defect in degradation of the walls, and after development the four microspores of the tetrad stage remain attached as mature, functional pollen (Preuss *et al*., 1994; Rhee and Sommerville, 1998; Rhee *et al*., 2003). Within the released microspores a gradient of cytoplasmic factors and the non-central positioning of the nucleus lead to an asymmetric mitotic division, which in turn produces a large vegetative cell and a smaller generative cell (figure 2c). A second mitotic division of the generative cell is needed to form two sperm cells. In *Arabidopsis*, this division takes place during pollen development in the anther, resulting in tricellular mature pollen with two sperm cells positioned within the vegetative cell (figure 2d). In species with bicellular pollen, like tobacco and lily, this second division takes place in the pollen tube. In most tricellular pollen species that have been examined, the two compact, but elongated, sperm cells are closely associated with the vegetative cell nucleus at the centre of the vegetative cell (reviewed in Mogensen, 1992; Dumas *et al.*, 1998; Lalanne and Twell, 2002). This association is called the male germ unit (MGU) and it is proposed to have a functional role in the controlled transport and delivery of the sperm cells to the embryo sac (Dumas *et al.*, 1984a,b, reviewed in Russell, 1996). Morphological screens of mutant *Arabidopsis* populations with the DNA stain DAPI to visualise the nuclei have led to the

identification of several mutants with defects in pollen mitosis I and/or pollen mitosis II. These gametophytic mutants include *solo pollen* (now called *tio* (*two-in-one*) Twell and Howden, 1998; reviewed in Twell, 2002), s*idecar pollen* (Chen and McCormick, 1996), *duo pollen* (Durbarry *et al.*, 2005; Rotman *et al.,* 2005) and *gemini pollen (gem)* (Park *et al.*, 1998). Symmetric division of the microspore in *gem1* leads to the formation of two cells that both had vegetative cell identity, as shown by the decondensed chromatin and the ability to express a vegetative cell–specific marker (Park *et al.*, 1998). *GEM1* encodes a microtubule-associated protein (MAP215 family), which is responsible for the correct positioning of the cytokinetic phragmoplast at pollen mitosis I (Twell *et al.*, 2002). The aberrant symmetric division might cause an altered partitioning of the cytoplasmic factors and alteration in cell fate, abolishing the formation of the generative cell and the second pollen mitosis. Other mutants showed a disruption or displacement of the male germ unit, like *limpet pollen* (Howden *et al.*, 1998), *germ unit malformed (gum)* and *male germ unit displaced (mud)* (Lalanne and Twell, 2002). Double mutant analysis of the *gum* and *mud* mutations showed that they affect the same process, with *GUM* acting earlier than *MUD* (Lalanne and Twell, 2002).

The pollen wall consists of an outer layer made up of sporopollenin, the exine, and an inner layer composed of pectin and cellulose, the intine (Owen and Makaroff, 1995). Both wall layers are formed by the microspores, and thus are of gametophytic origin, though the exine also contains sporophytic components derived from the tapetum. Synthesis of the exine layer starts with the deposition of primexine by microspores at the tetrad stage. At this stage, the distinct rough exine surface pattern is laid out, and the deposition of exine continues in the free microspores, while also material is deposited for the intine layer underneath the exine. At the stage of pollen mitosis II, components derived from the degenerating tapetum accumulate in the exine caverns and on the exine surface, forming a pollen coat or tryphine. Sporophytic mutants that affect the development of the tapetum and the production of the pollen coat generally result in male sterility, indicating the importance of the tapetum for pollen development and function (reviewed in Scott *et al.*, 2004).

The last step in pollen development involves partial dehydration of the pollen grains, which is largely mediated by evaporation upon anther dehiscence (figure 2e). With the identification of the male-gametophytic *Arabidopsis* mutant *raring-to-go*, in which mutant pollen under humid conditions can even germinate a tube while still in the anther, however, it was suggested that pollen dehydration is also partially regulated by the pollen itself (Johnson and McCormick, 2001). The mature, dehydrated pollen contain three longitudinal grooves that are the result of reduced exine deposition during the tetrad stage. These apertures are thought to be involved in pollen desiccation and rehydration, in grain shrinkage or swelling and in most species form the site of pollen tube emergence (Edlund *et al.*, 2004).

Pollen-pistil interactions

Arabidopsis is a self-fertile plant and mature pollen is transferred to the stigma of the same flower by direct contact. Upon pollen deposition, adhesion follows immediately relying on components within the pollen exine (Zinkl *et al.*, 1999). Pollen coat material accumulates between the pollen grain and the stigma, forming a 'foot' structure (Elleman and Dickinson, 1986; Elleman *et al.,* 1992), and lipids of the pollen coat facilitate pollen rehydration (figure 3a) (Zinkl and Preuss, 2000). Within minutes after pollen hydration, the pollen starts to germinate and changes occur in the pollen grain to support the extension of a single pollen tube, like the re-orientation of the cytoskeleton and the vegetative nucleus, and the polarized accumulation of secretory vesicles containing callose and other cell wall components at the site of pollen tube emergence (figure 3b) (reviewed in Edlund *et al.*, 2004). For successful fertilization, the germinated pollen tube has to penetrate the stigma and grow through the pistil during an event called the progamic phase. Two different processes can be distinguished in the progamic phase, that is 1) the process of cellular growth of the pollen tube (figure 3c) and 2) the process of directional growth towards the embryo sac within the ovules, which depends both on signalling cues from the pistil and on the ability of the pollen tube to respond to these signals (reviewed in Taylor and Hepler, 1997).

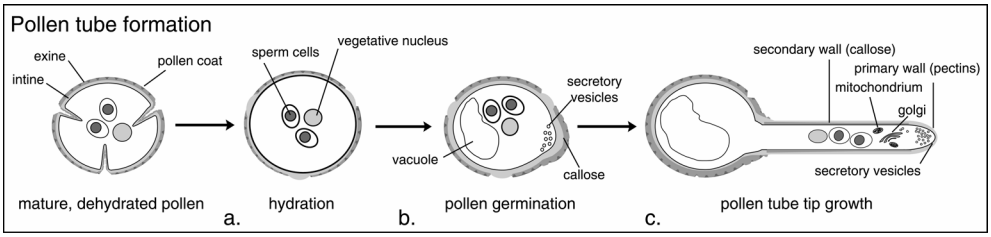

Figure 3: Pollen rehydration, germination and pollen tube growth
a. hydration b. polarization c. pollen tube tip growth

Pollen tube growth

Pollen tubes are fast growing cells and, like root hairs, extend exclusively at their apex by a specialized type of growth called tip growth. Most data on pollen tube growth were obtained from analysis of *in vitro* grown pollen tubes of tobacco, lily and poppy. Pollen tube elongation can be extremely rapid, with rates up to 1 cm/h, and growth is usually oscillatory. Underlying this type of growth are oscillations of polarized ion gradients and the turnover of cytoskeletal elements (actin microfilaments), as well as the process of exocytosis and endocytosis of secretory vesicles (reviewed in Feijo *et al.* 2001; Hepler *et al.*, 2001; Holdaway-Clarke and Hepler., 2003).

Though several different ions were shown to be involved in pollen germination and pollen tube growth, like H^+ (Deymer *et al.*, 2004; Robertson *et al.*, 2004), K^+

(Fan *et al.*, 2001; Mouline *et al.*, 2002; Sze *et al.*, 2004) and Cl^- (Zonia *et al.*, 2002), Ca^{2+} is the most important (reviewed in Hepler *et al.*, 2001). A tip-focused intracellular Ca^{2+} concentration gradient is essential for pollen tube elongation and for directional growth (reviewed in Holdaway-Clarke and Hepler, 2003, Wang *et al.*, 2004). This Ca^{2+} concentration is controlled both by Ca^{2+} mobilization from intracellular sinks (Malhó and Trawavas, 1996; Franklin-Tong *et al.* 1996; Malhó, 1998a; Xu *et al.*, 2005) and by extracellular influx through plasma membrane-localized Ca^{2+} channels (Pierson *et al.*, 1994, 1996; Malhó *et al.*, 1995; reviewed in Rudd and Franklin-Tong, 1999; Schiott *et al.*, 2004). Studies on pollen germination and pollen tube growth *in vivo* have shown that, already in the pollen grain, the cytosolic Ca^{2+} concentration is increased at the site of pollen tube emergence. Like shown *in vitro*, Ca^{2+} concentration oscillations are present at the pollen tube tip *in vivo*, and also in the stigma the cytosolic Ca^{2+} concentration is strongly increased during pollen rehydration, pollen tube protrusion and penetration of the papillar cell wall (Iwano *et al.*, 2004).

The cytoskeleton of the pollen tube consists of longitudinal actin cables, short actin filaments and microtubules. Though microtubules do not appear to have an essential role in pollen tube tip growth of angiosperms, it was shown to regulate the polarized growth and orientation of *Arabidopsis* root hairs through interactions with the Ca^{2+} gradient at the tip (Bibikova *et al.*, 1999). Longitudinal actin cables are involved in organelle movement within the pollen tube following the reverse fountain-like pattern of cytoplasmic streaming (Hepler *et al.,* 2001). A highly dynamic form of short F-actin bundles occurs in turn at the tip or in a collar just behind the tip (Staiger *et al.*, 1994; Kost *et al.*, 1999; Fu *et al.*, 2001). The levels of tip-localized F-actin oscillate in opposite phase with the oscillations found in pollen tube growth and in the Ca^{2+} concentration at the tip (Fu *et al.*, 2001).

In addition, pollen tube growth is dependent on a GTP binding protein, RHO OF PLANTS1 (ROP1), as is amply demonstrated by different approaches (Lin *et al.*, 1996; Lin and Yang, 1997; Li *et al.*, 1999; Kost *et al.*, 1999; reviewed in Gu *et al.*, 2003). Changes in the activated ROP1 concentration at the pollen tube tip was shown to affect both the intracellular Ca^{2+} concentration and the amount of tip F-actin. The current model is that ROP1 mediates F-actin assembly but also induces an increase in Ca^{2+} concentration. This increase in Ca^{2+} concentration in turn mediates disassembly of F-actin at the tip (Geitmann *et al*., 2000) that may involve various actin binding proteins (Kovar *et al.*, 2000; Yokota and Shimmen, 2000) and actin depolymerising factors (Fu *et al.*, 2001; Chen *et al.*, 2002; Gu *et al.*, 2003). The ROP1-mediated changes in calcium concentration presumably also lead to calcium dependent exocytosis of the secretory vesicles (Roy *et al.*, 1999; Wu *et al.*, 2001). Fusion of these Golgi-derived vesicles with the plasma membrane at the tip leads to extension of the plasma membrane and secretion of pectin components that form the primary pollen tube wall at the tip. The secondary, inner pollen tube

wall mainly consists of callose as well as cellulose, which are synthesized by callose synthases and cellulose synthases at the plasma membrane behind the tip (Ferguson *et al.*, 1998).

Pollen tube guidance

Preceding fertilization, the pollen tube needs to be guided from the stigma to the embryo sac. The pollen tubes grow intercellularly through the stigma and style, and grow onto the septum surface in the ovary. Components of the transmitting tract and arabinogalactan proteins of the extracellular matrix that are secreted by cells of the septum were proposed to guide the pollen tubes to the ovules (Lennon *et al*., 1998; Johnson and Preuss, 2002; Sanchez *et al*., 2004). Gamma-amino butyric acid (GABA) is one of the identified mediators of pollen tube guidance in the pistil (Palanivela *et al.*, 2003). A gradient of GABA is formed within the ovary by its degradation by the transaminase POLLEN PISTIL INTERACTION2 (POP2). In *pop2 Arabidopsis* mutants, pollen tube growth is strongly affected in the presence of GABA, and pollen tubes fail to adhere to the ovules in mutant plants (reviewed in Ma, 2003). Also nitric oxide (NO) has been found to regulate pollen-tube growth in *Lilium longiflorum*, possibly through a cGMP transduction pathway (Prado *et al.*, 2004). Other female sporophytic components regulating pollen tube guidance were found in tobacco, where pollen tubes were suggested to be guided to the ovules by a gradient in glycosylation of the arabinogalactan protein TTS (transmitting-tract-specific protein) within the extracellular matrix of the style. In addition to a guidance signal, TTS was also suggested to promote pollen tube elongation by providing sugar moieties, as pollen tubes appear to be able to deglycosylate TTS (Wu *et al.*, 1995; Cheung *et al.*, 1995).

Analysis of pollen tube growth in the ovaries of *Arabidopsis* sporophytic and gametophytic mutants showed that the embryo sacs themselves also attract pollen tubes (Hülskamp *et al.*, 1995; Ray *et al.*, 1997; Shimizu and Okada, 2000). This female gametophyte guidance can be subdivided into funicular and micropylar guidance (reviewed in Weterings and Russell, 2004) and cell ablation experiments in *Torenia fournieri* demonstrated that the attraction of pollen tubes is at least partly controlled by the synergid cells (Higashiyama *et al.*, 2001). Typically, only one pollen tube is present on the funiculus of the ovule to enter the embryo sac. Mutant analysis of the *Arabidopsis* female gametophytic *magatama* mutants suggested that this is controlled by the female gametophyte to prevent polyspermy, though it might also involve repulsion signals between pollen tubes (Shimizu and Okada 2000). Other evidence for female control of male gamete delivery during fertilization comes from the mutants *feronia* and *sirène* (Huck *et al.*, 2003; Rotman *et al*., 2003). In *feronia,* pollen do not burst to release the sperm cells within mutant embryo sacs, but continue to grow inside the embryo sac (Huck *et al*., 2003). Also live imaging of fertilization within the *sirène* embryo sacs showed that wild-type

pollen tubes did not stop their growth and did not deliver their contents in *sirène* embryo sacs (Rotman *et al.*, 2003). Multiple pollen tubes were found near a single *sirène* embryo sac, suggesting that the attraction of pollen tubes to the micropyle was not arrested upon penetration of one of the synergid cells (Rotman *et al.*, 2003).

Cell wall components and the activity of glycosyl transferases

Pollen tubes are fast growing cells and therefore need large amounts of cell wall components to form the pollen tube wall, within a relatively short time. Unlike most plant cells, in which growth occurs through modifications of the existing cell wall and the insertion of new material throughout its surface (Cosgrove, 2000), the pollen tube grows strictly at the apex. The pollen tube wall consists of the polysaccharide polymers cellulose, callose, hemicellulose and pectin, and also contains glycoproteins and proteoglycans (Costa *et al.*, 2002). Several monosaccharide transporters (like STP2, 6, 9 and 11 in *Arabidopsis* and PMT1 in *Petunia*) are specifically expressed in pollen, suggesting a role in the supply of sugar substrates for the synthesis of these cell wall components (Ylstra, 1998; Truernit *et al.*, 1999; Scholz-Starke *et al.*, 2003; Schneidereit *et al.*, 2003, 2004). Enzymes that mediate the transfer of nucleotide-activated sugars to proteins or other polysaccharides are the glycosyl transferases (Keegstra and Raikhel, 2001). In *Arabidopsis,* 415 glycosyl transferases have been classified in the Carbohydrate-Active enZYmes (CAZy) database and these are divided into 25 different families based on amino acid sequence similarities and specificity for sugar residue and acceptor molecule (Henrissat and Davies, 2000). With the exception of the cytoplasmic soluble glycosyl transferases involved in glycolipid (secondary metabolites) synthesis and the multi-membrane spanning cellulose and callose synthases that are located at the plasma membrane, most other glycosyl transferases are localized in the secretory pathway (the endoplasmatic reticulum (ER) and Golgi or dictyosomes) and participate in the synthesis of hemicellulose, pectin, and glycoproteins. This latter group of glycosyl transferases consists of single membrane-spanning proteins, with the transmembrane domain at the amino-terminal side of the protein (the so-called type II transmembrane protein orientation) and the large carboxy terminus with the catalytic domain facing the ER or Golgi lumen (figure 4a). Non-cellulosic polysaccharides and cell surface–associated glycoproteins contain a number of different sugars, such as galactose, arabinose, fucose, xylose, mannose, rhamnose, glucose, galacturonic acid, and glucuronic acid, and several *Arabidopsis* Golgi-localized nucleotide sugar transporters have been isolated, which transfer the cytosolic nucleotide sugars to the Golgi lumen to be used in glycosylation reactions (Baldwin *et al.,* 2001; Handford *et al.,* 2003). The subsequent actions of various glycosyl transferases

during hemicellulose, pectin and glycoprotein synthesis result in a linear and/or branched polymer composed of monosaccharides linked to one another with extraordinary precision (figure 4b-d). The different glycosyl transferases are localized in the secretory pathway and Golgi cisternae in the same sequence in which they act.

Cellulose and callose

Cellulose and callose are β-linked D-glucan polymers and differ from each other in the way the glucose residues are linked to form a polymer. Cellulose consists of long microfibrils of (1→4) β-linked D-glucose chains (Brown *et al.*, 1996). The inner part of the fibril is crystalline, each glucose unit rotated 180° to form a crystal structure. Callose on the other hand, is a (1→3) β-linked D-glucan, and consists of non-linear polymers. Cellulose synthases and callose synthases are glycan synthases grouped into the glycosyl transferase family 2 and are organized in plasma membrane-localized rosettes composed of six symmetrical catalytic units (Richmond, 2001). Each rosette of cellulose synthases produces a single microfibril, and many rosettes together produce the cellulose fibril. Unlike cellulose synthases, callose synthases need a UDP glycosyl transferase (UGT1) to bind UDP-glucose (Verma and Hong, 2001). UGT1 in turn, was shown to react with ROP1, suggesting also that callose synthase activity in pollen tubes is regulated by this protein (Verma, 2001). In pollen tubes, callose- and cellulose synthases are located in the plasma membrane flanking the tip (Ferguson *et al.*, 1998; Doblin *et al.*, 2001). Callose constitutes over 80% of the total pollen tube wall components (Schlüpmann *et al.*, 1993) and is localized in the secondary, inner pollen tube wall, which also contains small quantities of cellulose. Plugs of callose are present in longer pollen tubes, sealing of the older parts of the pollen tubes and keeping the cytoplasm and organelles at the front region of the pollen tube. A cellulose synthase-like glycosyl transferase (AtCSLA7) was shown to be essential for pollen tube growth, as well as for embryo development, suggesting that this putative β-glycosyltransferase is essential for cell wall structure both in pollen and potentially in signalling between embryo and endosperm in the sporophyte (Goubet *et al.*, 2003).

Hemicellulose and pectin

The noncellulosic polymers hemicellulose and pectin are synthesized by glycosyl transferases located in the different compartments of the Golgi apparatus. Hemicelluloses can contain various sugar residues and are divided into xyloglucans, arabinoxylans, xylans, glucomannans and galactomannans. Xyloglucan is the principle component of hemicellulose in the primary walls of dicots, and consist of a glucan backbone with repeated side chains containing xylosyl, galactosyl and fucosyl residues (figure 4b). The *Arabidopsis* mutants *mur1,*

mur2 and *mur3* have a defect in xyloglucan synthesis, which causes a dwarfed phenotype, but no defects in pollen germination or tube growth have been described for these mutants. The *mur1* mutant has a defect in L-fucose synthesis (Zablakis *et al.,* 1996), and the *MUR2* and *MUR3* genes code for xyloglucan galactosyltransferases (Vanzin *et al.,* 2002; Madson *et al.,* 2003).

Pectin is synthesized within the Golgi apparatus by polymerization of UDP-galacturonic acid monomers to form the homopolymer backbone homogalacturonan or, in combination with rhamnose residues, the heteropolymer backbone rhamnogalacturonan I (figure 4d). Methyl groups are added to the pectin backbones, resulting in acidic methyl-esterified pectins, and branches consisting of different sugar residues like arabinan and galactan are added as side chains. Rhamnogalacturonan II is a modification of the homogalacturonan backbone by the addition of sugar side chains containing rhamnose and rare sugar residues like KDO (Costa *et al.,* 2002). Considering the complex structure of pectin, over 50 different glycosyl transferases are estimated to be required for synthesis of the pectin polymers alone. Pectin and hemicellulose are transported in secretory vesicles to the tip of the pollen tube, and released to form the primary (outer) pollen tube wall. In petunia pollen tubes, polygalacturonate 4-α-galacturonosyltransferase, a pectin synthase involved in the synthesis of the pectin backbone, has been isolated (Akita *et al.*, 2002). Pectin constitutes ~10% of the pollen tube wall weight and perturbation of the pectin transport during pollen tube germination results in the arrest of pollen tube growth (Geitmann *et al.*, 1996).

Pectin of both the pollen intine layer and the primary pollen tube wall is deposited at the wall by secretory vesicles as highly methyl-esterified pectin (Li *et al.*, 1994; Geitmann *et al.*, 1995), and can be de-esterified by pectin methylesterases within the pollen tube wall. The presence of both pectin methylesterases and pectin methylesterase inhibitors in pollen suggests that this pectin demethylation is dynamically regulated (Jiang *et al.,* 2005; Wolf *et al.*, 2003). Immuno-localization studies have shown that the intine layer of mature, unhydrated pollen grains mainly contains methyl-esterified pectin (recognized by the JIM7 antibody), while the intine of germinating pollen grains is de-esterified to a larger degree (recognized by the JIM5 antibody) (Van Aelst, 1992; Li *et al.*, 1995; Hasegawa *et al.*, 2000; Suarez-Cervera *et al.*, 2002; Castells *et al.*, 2003; Abreu and Oliveira, 2004). Also within the growing pollen tube, the wall at the tip contains methyl-esterified pectin, while the older, outer regions of the pollen tube wall are increasingly de-esterified (Li *et al.*, 1992, 1994; Lennon and Lord, 2000). Unlike the esterified pectins, de-esterified polysaccharides can cross-link with Ca^{2+} and form rigid insoluble gels (Jarvis 1984; Carpita and Gibeaut, 1993; Gibeaut and Carpita, 1994). Pectin modification probably alters cell wall strength to confer plasticity for pollen tube emergence at the intine and cell extension at the tube tip (Steer and Steer, 1989; Carpita and Gibeat, 1993).

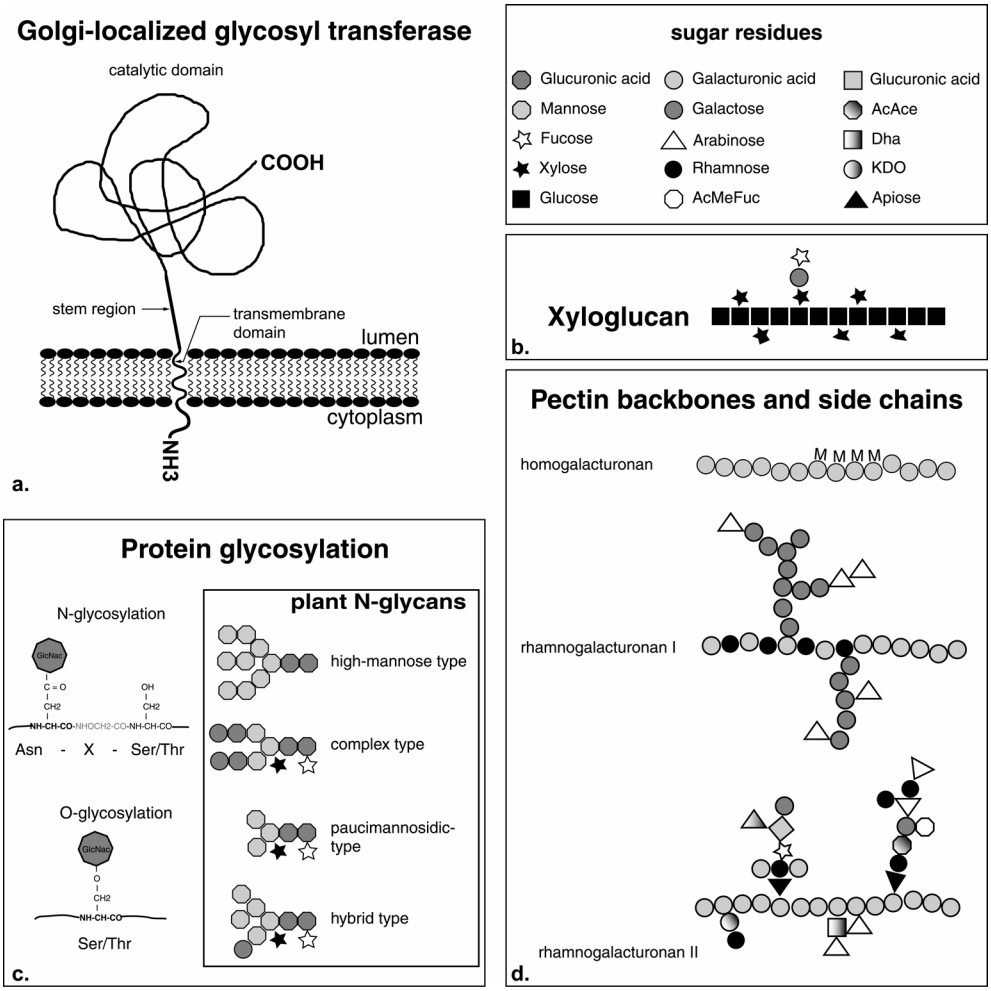

Figure 4: Golgi-resident glycosyl transferase and its products
Schematic representation of a. a Golgi-resident type II transmembrane glycosyl transferase (adjusted from Keegstra and Raikhel, 2001) and its possible products. b, xyloglucan (Costa *et al.,* 2002), c, protein N-glycans (Lerouge *et al.,* 1998), and d. pectin (adjusted from Perez *et al.,* 2003).

Glycoproteins and proteoglycans

Glycosylation of proteins in plants and animals can be divided into O-glycosylation and N-glycosylation, based on the element on the acceptor molecule used to bind the sugar residue (figure 4c). N-glycosylation of proteins starts during protein synthesis at the ER membrane with the attachment of an oligosaccharide sugar tree to the amide nitrogen (N) atom at the side chain of the asparagine amino acid present in the N-glycosylation site Asn-X-Ser/Thr (Kornfeld and Kornfeld, 1985). This oligosaccharide is not directly synthesized on the protein, but

is synthesized on the carrier dolichol phosphate, which is a very long lipid embedded in the membrane of the ER. Dolichol phosphate acquires an oligosaccharide unit consisting of two N-acetylglucosamine, nine mannose, and three glucose residues ($Glc_3Man_9(GlcNac)_2$) by the sequential addition of monosaccharides in the ER. This involves the activity of at least two different N-acetylglucosaminyl transferases: N-acetylglucosaminyltransferase I is involved in the initial glycosylation of the dolichol carrier, and N-acetylglucosaminyltransferase II (GnTII) links GlcNAc from UDP-GlcNAc to mannosyl residues in the synthesis of complex N glycans (reviewed in Costa *et al.,* 2002). The oligosaccharide then is transferred *en bloc* to the growing polypeptide chain on the luminal side of the ER membrane. Only when the protein is folded correctly, the three glucose residues and one of the mannoses are removed from the N-linked glycan, an indication that the glycoprotein can be transported to the Golgi for further processing. Here, other sugar residues are removed and added in many different ways to form different N-glycan patterns that can be classified into four categories: high-mannose-type, complex-type, paucimannosidic-type and hybrid-type N-glycans (figure 4c) (Lerouge *et al.*, 1998). Fucose and xylose residues are besides mannose and N-acetylglucosamine the most frequently used monosaccharides for N-glycan modification. Fucosylated N-glycans are thought to be involved in cell-to-cell communication and recognition. During pollen development and pollen tube growth an increase in the activity of α(1,4) fucosyltransferase was found, suggesting a role for this enzyme and N-glycosylation in these processes (Joly *et al.,* 2002).

O-glycosylation of proteins takes place in the Golgi apparatus and is thought to affect protein activity, stability, and/or localization. The sugar residues are covalently linked to the oxygen (O) atom at the side chains of serine, threonine or hydroxyproline residues (figure 4c). In *Arabidopsis*, *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc is transferred to the Ser/Thr residues of proteins by the *O*-linked *N*-acetylglucosamine transferases SECRET AGENT (SEC) and SPINDLY (SPY). Although the *sec* mutant plants did not exhibit obvious phenotypes, double mutants with *spy* showed a reduction in the *sec spy* gamete transmission rate, and double-mutant embryos aborted at various stages of development. This shows that O-glycosylated proteins are required during gametogenesis and embryogenesis (Hartweck *et al.,* 2002). Proteoglycan synthesis also involves O-glycosylation, but proteoglycans differ from O-glycoproteins in the quantity and length of the sugar moieties, which constitutes over 90% of the total weight of the molecule. Arabinogalactan proteins (AGPs) constitute a specific group of proteoglycans, and have arabinosyl and galactosyl residues as the major sugar moieties (Costa *et al.*, 2002).

Both N-linked glycoproteins and AGPs have been reported in the secondary pollen tube wall, and are likely to play a role during pollen tube wall construction and thus pollen tube growth (Li *et al.*, 1997). The pattern of glycoproteins present

in pollen changes during pollen development (Rihova *et al.*, 1996; Hruba and Tupy, 1999). Examples of these glycoproteins are the pollen-specific 66 kDa and 69 kDa glycoproteins of tobacco (Čapkova *et al.*, 1994; Fidlerova *et al.*, 2001). Glycosylation of these abundant proteins seems to play an important role during pollen germination, since treatment with tunicamycin, an inhibitor of N-glycosylation, can inhibit pollen tube growth in tobacco (Čapkova *et al.*, 1997). The 69 kDa glycoprotein is encoded by the NTP303 gene that is specifically expressed in pollen (Weterings *et al.*, 1992), and is localized in the wall, the vegetative cell plasma membrane and, in a lower extent, in the callose plugs of pollen tubes (Wittink *et al.*, 2000). Analysis of doubled haploid antisense plants, in which the expression of NTP303 was strongly reduced in pollen, showed an arrest in pollen tube growth *in vivo*, while *in vitro* pollen tube growth appeared completely normal (De Groot *et al.*, 2004). This suggested that the NTP303 protein is important during pollen-pistil interactions and presumably is involved in perceiving signalling cues from the pistil. Also immuno localization studies of a pollen-specific hydroxyproline-rich glycoprotein with an extensin-like domain (PEX1) showed localization in the intine layer of mature pollen and in the inner layer of the pollen tube wall (Rubinstein *et al.*, 1995a,b). The localization of these glycoproteins in the pollen tube wall is consistent with a function in pollen wall construction, or alternatively they may be involved in the interaction with the pistil during the progamic phase. Analysis of homozygous antisense plants of two other pollen-specific glycoproteins, LAT52 of tomato and SHY, a leucine-rich repeat protein of petunia, showed that these proteins are important for pollen germination and pollen tube growth (Musschietti *et al.,* 1994; Guyon *et al.,* 2004). Two hybrid screens showed that LeLAT52, like the pollen LeSHY and the stigma-expressed LeSTIG1, interacts with the pollen-specific receptor kinase LePRK1 and LePRK2 (Tang *et al*., 2002, 2004). The LAT52-PRK2 and SHY-PRK2 interaction is thought to activate a signaling cascade required to initiate pollen tube growth (Tang *et al*., 2002, 2004). *In vitro* binding assays showed that upon germination, LAT52 might be replaced by LeSTIG1, a protein suggested to promote pollen tube growth*,* suggesting that LePRK1 and LePRK2 are involved in mediating pollen–pistil interactions at various levels (Tang *et al*., 2004).

Immuno localization of AGPs showed that they were present in the vegetative cell membrane near the intine and in the sperm cells of mature *Arabidopsis* pollen (Van Aelst *et al.*, 1992), as well as in tobacco pollen (Li *et al.*, 1995, Ferguson *et al.*, 1999). Also in pollen tubes AGPs have been detected, though this appears to differ between species and is dependent on the protocol used for immuno detection (reviewed in De Graaf, 2001). In lily, AGPs are located at the pollen tube tip (Jauh and Lord, 1996; Mollet *et al.*, 2002) and perturbation experiments with Yariv phenylglycosides (that bind and precipitate AGPs) showed that AGPs are essential for pollen tube growth in some species (Roy *et al.*, 1998, Mollet *et al.*,

2002) and might also be involved in pollen germination (Abreu and Oliveira, 2004). However, pollen AGPs are considered of no importance for tip growth in tobacco and *Arabidopsis* (Jauh and Lord, 1996; Roy *et al*. 1998). Several other AGPs similar to TTS (Cheung *et al.*, 1995; Wu *et al.*, 1995) have been found to be expressed within the pistil, like the galactose-rich style glycoprotein (GaRSGP, Lind *et al.*, 1994), PELPIII (Bosch *et al.*, 2001), and a 120 kDa glycoprotein (Sommer-Knudsen *et al.*, 1996), though their effect on pollen tube growth is not clear.

Glycolipidic secondary metabolites

Although secondary metabolites are not real components of the pollen tube wall, some of them do play a role in pollen germination. Most secondary metabolites are lipidic compounds that are glycosylated within the cytoplasm by soluble O-glycosyl transferases called glucosyl transferases (Costa *et al.*, 2002). Plant secondary metabolism glucosyl transferases are grouped into glycosyl transferase family 1 and include anthocyanin and flavonol glucosyl transferases. Flavonol 3-*O*-galactosyltransferase (F3GalTase) is a pollen-specific enzyme which glycosylates the flavonols required for pollen germination in petunia (Mo *et al.*, 1992; Taylor and Jorgensen, 1992). F3GalTase forms flavonol 3-*O*-galactosides, which are subsequently glucosylated by a glucosyl transferase to form the pollen-specific conjugates of kaempferol and quercetin (Vogt and Taylor, 1995; Miller *et al.*, 1999). In *Arabidopsis* however, pollen germination was not affect in the *tt4* mutant, which is mutated in the gene coding for chalcone synthase, the first enzyme involved in flavonoid synthesis, suggesting that flavonoids are not universally required for pollen germination and fertilization (Burbulis *et al.*, 1996; Ylstra *et al.*, 1996).

Genome wide analysis of pollen expressed genes

Pollen development and pollen tube growth are regulated by gametophytically expressed genes, as well as by genes expressed in the surrounding sporophytic tissues. Genes expressed in the vegetative pollen cell are necessary for proper pollen development and germination, pollen tube growth and guidance towards the egg cell, while genes expressed in the sperm cells are considered to be involved in gamete recognition and early fertilization events (Russell, 1985; Xu *et al.*, 2002; Engel *et al.*, 2003; Da Costa *et al.*, 2003). The first studies on quantitative gene expression in pollen were based on mRNA hybridisation kinetics and isozyme analysis of pollen and sporophytic tissues of *Trandescantia* and maize. From these studies it was estimated that ~20.000 different genes are expressed in mature pollen. 60 to 72% of the isozymes analyzed were expressed in both gametophytic and sporophytic tissues, and ~10% of the isozymes found in pollen were pollen-specific (Willing and Mascarenhas, 1984; Willing *et al.*, 1988). Recent micro-array

analyses of the pollen transcriptome in *Arabidopsis* have shown that these estimated frequencies are quite accurate (Honys and Twell, 2003, 2004; Becker *et al.*, 2003). Data of the ATH1 micro-array showed that 56% of the genes on the chip were expressed both in pollen and sporophytic tissues, and that 9.7% of the genes expressed in pollen were pollen-specific (Honys and Twell, 2004). The ATH1 array covers 80.7% of the ~28.000 protein-coding genes in the *Arabidopsis* genome (Yamada *et al.*, 2003) and calculations based on the pollen transcriptome data gave an estimate of 17323 pollen-expressed genes, of which 1683 genes are specifically expressed in pollen (Honys and Twell, 2004). Mature pollen showed a very divergent expression profile, clearly different from that of the sporophytic transcriptome (Honys and Twell, 2003, 2004; Becker *et al.*, 2003). Qualitative analysis of the pollen transcriptome showed that most pollen-specific genes were very highly expressed in mature pollen (Honys and Twell, 2004). Not surprisingly, these pollen-specific genes were preferentially involved in cell wall metabolism, signalling and cytoskeleton organisation, while genes related to photosynthesis or translation were underrepresented (Honys and Twell, 2003, Becker *et al.*, 2003; Lee *et al.*, 2003, Da Costa-Nunes *et al.*, 2003). The identification of more than 1000 pollen-specific genes, as well as genes that show interesting pollen development expression profiles, provides a new tool for pollen developmental research. Reverse genetic mutational analysis of these genes without a doubt will give a lot of extra information on the molecular regulation of sexual plant reproduction.

Gametophytic mutants can be identified by 'segregation distortion'

Notwithstanding the vast amount of interesting genome-wide pollen expression data, genes important for pollen development and function that do not show interesting transcription profiles, as they are expressed at low level or are not tissue-specific, will not be identified in these assays. They can only be isolated using forward genetics, by mutant analysis and subsequent cloning of the mutated gene. In *Arabidopsis*, most mutant populations nowadays are made by insertional mutagenesis rather than by treatment with EMS or radiation. *Agrobacterium tumefaciens* based plant transformation leads to the random insertion of a piece of the Ti (tumour-inducing) plasmid DNA, the so-called transfer-DNA (T-DNA), within the plant genome. Besides the advantage of stable integration of transgenes within the plant DNA, this T-DNA insertion can also cause a mutation of an endogenous gene when inserted in the promoter or coding regions. Insertional mutagenesis has the advantage that the mutation is tagged by the inserted T-DNA, and the presence of the T-DNA can be followed in the progeny by tracing a selectable marker carried on the T-DNA. In addition, the mutated gene can be identified relatively easily by molecular biological techniques like TAIL-PCR or plasmid

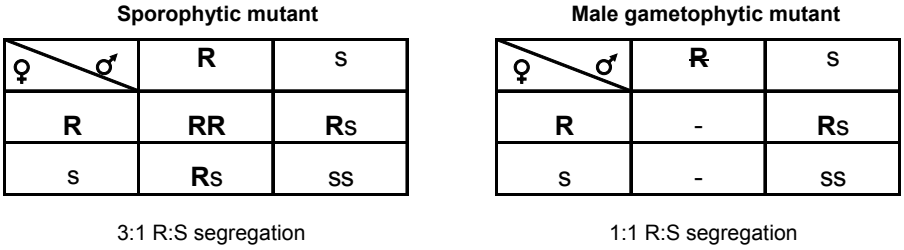

Figure 5: Segregation distortion of a gametophytic mutation
R, mutated gene of a T-DNA insertional mutant represented by the antibiotic resistance gene on the T-DNA. s, wild-type gene

rescue, compared to laborious map-based cloning, in plants showing an interesting distorted phenotype.

Genetic screens to identify mutants of male gametophytic genes essential for pollen development and for the progamic phase are based on screening for distorted segregation ratios of the selectable marker (usually conferring antibiotic resistance) carried by the T-DNA or transposon element (Howden *et al.* 1998; Bonhomme *et al.,* 1998a; Lalanne *et al.*, 2004a). Similarly, segregation distortion of linked markers on the multiple marker chromosome I in an EMS mutagenized *Arabidopsis* population was used to identify gametophytic mutants (Grini *et al.*, 1999). Normally, the T-DNA insertion of a hemizygous sporophytic mutant leads to a 3:1 segregation of the antibiotic resistance gene, according to the Mendelian laws of segregation. Plants with a gametophytic mutation, however, fail to transmit the T-DNA insertional mutation to the offspring through the affected gamete. This defect in T-DNA transmission will result in a segregation of 1:1 (or less) resistant to sensitive seedlings, depending on the severity of the mutation (figure 5) (Feldmann *et al.*, 1997; Howden *et al.*, 1998). Using the phenotype of 'segregation distortion', numerous gametophytic mutants have been isolated so far (Howden *et al.*, 1998; Bonhomme *et al.*, 1998a,b; Christensen *et al.*, 1998; Procissi *et al*., 2001; Johnson *et al.*, 2004; Lalanne *et al.*, 2004b). On average 0.9% of the insertional mutants screened thus far were male gametophytic mutants (Howden *et al.*, 1998; Bonhomme *et al.*, 1998a; Lalanne *et al.*, 2004a), which is rather low, considering that approximately 6% of all genes are expressed specifically in pollen (Honys and Twell, 2004). However, functional redundancy of genes could hamper their isolation through this genetic screen. As most of the mutant populations screened for gametophytic mutants were generated by 'floral dip' *Agrobacterium*-mediated transformation, and the primary target of T-DNA integration using this method is the embryo sac, the recovered female gametophytic mutant plants using segregation distortion analysis is even much less (Ye *et al.*, 1999; Bechtold *et al.*, 2000; Desfeux *et al.*, 2000). However, a recent screen of 24.000 Ds insertion lines

(with the Ds transposable element inserting in sporophytic cells) resulted in 130 female gametophyte mutants (Pagnussat *et al.*, 2005), and sequence analysis of the disrupted genes showed that they were involved in protein degradation, cell death, signal transduction and transcriptional regulation, processes important for embryo sac development, fertilization and early embryogenesis (Pagnussat *et al.*, 2005).

Male gametophytic genes involved in the progamic phase

Molecular characterization of male gametophytic mutants so far revealed the identification of 25 genes involved in the progamic phase, which are shown in table 1. The haploid disrupting *hapless (hap)* male gametophytic and male and female gametophytic mutants were identified from a T-DNA insertional population in the *quartet* background, the T-DNA carrying *LAT52::GUS* to facilitate mutant phenotype analysis (Johnson *et al.*, 2004). Two pollen tube growth mutants, *kinky pollen (kip)* and *poky pollen tube (pok)* were identified in the Versailles T-DNA population collection (Procissi *et al.*, 2003; Lobstein *et al.*, 2004), and ten male gametophytic *seth* mutants as well as ten male and female gametophytic *ungud* (*ung*) mutants were isolated from a Ds transposon population (Lalanne *et al.*, 2004b). The majority of these mutants did not give rise to homozygous mutant progeny, suggesting that either fertilization does not take place or that the homozygous mutation is embryo lethal.

Several identified genes seemed to code for proteins involved in cell wall synthesis. These predicted proteins were an Arabinose-5-phosphate isomerase (*seth 3*), involved in the synthesis of the rare sugar residue KDO, that in turn is required for synthesis of the pectin backbone rhamnogalacturonan-II, two phosphatidylinositol-glycan synthases (*seth1*&*2*), involved in GPI anchor biosynthesis, and a sucrose transporter (*hap3*), that could function in the supply of monosaccharide for the synthesis of the polysaccharide cell wall components. Other genes involved in cell wall synthesis and modification were found by reverse genetic mutant analysis and encode the cellulose synthase-like protein AtSCLA7 (Goubet *et al.*, 2003) and the pectin methylesterase VANGUARD1 (VGD1) (Jiang *et al.*, 2005). *Vgd1* mutants show a severe reduction in pollen tube growth within the pistil, and *vgd1* pollen tubes burst *in vitro* (Jiang *et al.,* 2005). The loss of VGD1 function effects the formation of Ca^{2+} pectate gel in the pollen tube wall, resulting in a reduction in strength of the pollen tube wall.

Gene products that could to be involved in Golgi vesicle transport and delivery of the secretory vesicles to the pollen tube tip membrane were identified from the *hap6* and *pok* mutants, and show homology to a ribophorin II-like protein and Vps52p/SAC2 protein, respectively. Also reverse genetic studies on syntaxins, known to be involved in vesicle transport to the membrane, showed that these proteins have an essential, non-redundant, role in pollen germination (Sanderfoot

et al., 2001). Other identified genes seem to encode proteins involved in various different processes such as briefly mentioned hereafter. The affectd geneproducts of *seth6, seth7* and *ung6* are presumably involved in cell signalling. The UNG6 gene encodes a calcium dependent protein kinase, showing the importance of calcium for pollen tube growth. The identification that the calmodulin-binding protein NO POLLEN GERMINATION (NPG1) is essential for pollen germination suggests interaction with the Ca^{2+} pathway (Golovkin and Reddy, 2003, Safadi *et al.*, 2000). The shaker pollen inward K^+ channel (SPIK) is also required for pollen germination (Mouline *et al.*, 2002), which is consistent with the result that *in vitro* pollen germination is dependent on the extracellular K^+ concentration (Fan *et al.*, 2001), as well as with the expression of cation/H^+ exchanger genes in pollen (Fan *et al.*, 2001; Sze *et al.*, 2004). Other genes could be related to energy production (*hap11*), gene expression (*seth4, hap1 and hap4*) and metabolism (*ung10*). Some of the genes isolated (from the *seth4, 5, 8* and *10, hap1, 2, 4* and *8* and *ung9* mutants) do not show homology to known genes or no protein function is established and thus may involve novel signalling pathways worth analyzing.

Sometimes, T-DNA insertion in the plant genome causes genomic deletions or translocations of part of the chromosomes and the molecular aberrations of these mutants are generally very difficult to analyse. In one male gametophytic mutant, *halfman,* showing a defect in pollen development and progamic phase, a single copy of the *Ds*E transposon element was inserted into the second exon of the RLK5 gene adjacent to a large genomic deletion (Oh *et al.*, 2003). The authors identified 38 genes within the 150 kb deleted region, one or more of which have important function(s) for pollen maturation and seed development. Another example for difficulties that can arise in the identification of T-DNA tagged male gametophytic mutants is the presence of redundant pollen genes. An example for redundancy in pollen germination is the reverse genetics mutant analysis of members of the Apyrase family, enzymes that hydrolyze nucleoside tri- and diphosphates. Two T-DNA knockout mutants, *apy1* and *apy2*, were analyzed and though the single mutants showed only some reduction of male transmission, the male transmission was completely blocked in the double mutants (Steinebrunner *et al.,* 2003). Such redundant genes are impossible to isolate from segregation distortion analysis alone, and a combination of reverse and forward genetics will be needed for a complete analysis of genes involved in the pollen progamic phase. Protocols from different laboratories for large-scale pollen isolation and analysis have recently been combined and published in order to facilitate *Arabidopsis* pollen research (Johnson-Brousseau and McCormick, 2004).

Table 1: Male gametophytic genes involved in the progamic phase

Mutant	M/F affected	TE male (%)	mutant phenotype	Gene
pollen germination				
forward genetics				
seth1	M	0	strongly reduced pollen germination, abnormal callose deposition	At2g34980
seth2	M	1,6	strongly reduced pollen germination, abnormal callose deposition	At3g45100
seth6	M	1,1	strongly reduced pollen germination (and tube growth)	At2g57860
seth7	M	0,7	strongly reduced pollen germination (and tube growth)	At2g41930
halfman	M	6,6	aborted pollen at mid bicellular stage (35%) and defect in progamic phase (12%)	At4g28490 till At4g28830
reverse genetics				
syp21 and syp42	M	NT	reduced pollen germination	At5g16830 or At5g26980
No pollen germination1	M	NT	no pollen germination	At2g43040
SPIK	M	reduced	reduced pollen germination, shorter and slower tube growth	At2g25600
apy1	M	69,8	no effect when *APY2* intact	At3g04080
apy2	M	34,3	reduced male transmission	At5g18280
apy1+apy2 double mutant	M	NT	reduced pollen germination, no double homozygous mutants	At3g04080 At5g18280
pollen tube growth				
forward genetics				
seth3	M	0	progamic phase mutant	At3g54690
seth4	M	0	progamic phase mutant	At4g34940
seth5	M	0,8	progamic phase mutant	At4g00800
ungud6	M&F	11,1	progamic phase mutant	At4g04710
ungud9	M&F	4,2	progamic phase mutant	At2g34680
ungud10	M&F	10,2	progamic phase mutant	At3g54090
hap13	M	0,0	short pollen tube growth, failure to exit style	At1g60780
hap15	M	0,9	short pollen tube growth, failure to exit style	At1g20200
hap3	M	1,3	short pollen tube growth, failure to exit style	At1g66570 or At1g66580
hap6	M	0,0	short pollen tube growth, failure to exit style	At4g21150
hap8	M&F	5,5	short pollen tube growth, failure to exit style	At5g56250
kinky pollen	M	<16	twisted and/or branched pollen tubes (tip growth defect)	At5g49670 or At5g49680
poky pollen tube	M	0,92	short pollen tubes and slow growth	At1g71270
hap1	M	1,8	pollen tubes fail to leave septum	At1g02140
hap2	M	0,7	chaotic pollen tube growth in ovary	At4g11720
hap4	M&F	20,3	chaotic pollen tube growth in ovary	At3g52590
seth9	M	18,5	slow pollen tube growth	At5g42250
seth8	M	29,7	slow pollen tube growth	At5g13650
seth10	M	14,8	slow pollen tube growth	At2g03070
hap11	M	22,0	no micropyle penetrance	At5g47020 or At5g47030
reverse genetics				
vanguard	M	2,4%	unstable pollen tubes and slow pollen tube growth	At2g47040

M/F male or female gametophyte affected by the mutation. TE, T-DNA transmission efficiency. TE=R/Sx100%. NT, not transmitted.

other half of table 1

Predicted protein	Possible role in pollen function	References
Phosphatidylinositol-glycan synthase C (PIG-C)	GPI anchor synthetic pathway, protein anchoring, cell wall synthesis	Lalanne *et al.,* 2004a,b
Phosphatidylinositol-glycan synthase A (PIG-A)	GPI anchor synthetic pathway, protein anchoring, cell wall synthesis	Lalanne *et al.,* 2004a
RPT2/NPH3-like protein	pollen germination, signalling	Lalanne *et al.*, 2004b
Ser/Thr protein kinase	pollen germination, signalling	Lalanne *et al.*, 2004b
38 predicted genes in deleted region	unknown	Oh *et al.,* 2003
SYP2 and SYP4 (AtTLG2a) syntaxins	Fusion of transport vesicles to target membranes, cell wall biosynthesis	Sanderfoot *et al.,* 2001
NPG1, calmodulin-binding protein	involved in Ca^{2+} mediated pathway, precise function unknown	Golovkin and Reddy, 2003
Shaker Pollen Inward K^+ channel	pollen tube K^+ influx and homeostasis	Mouline *et al.,* 2002
Apyrase	hydrolysation of nucleoside tri- and diphosphates, ATP mediated export?	Steinebrunner *et al.,* 2003
Apyrase	"	Steinebrunner *et al.,* 2003
members of two of the three apyrase families	"	Steinebrunner *et al.,* 2003
Arabinose-5-phosphate isomerase	Kdo synthesis required for pectin synthesis and cell wall biosynthesis	Lalanne *et al.,* 2004b
armadillo repeat containing protein	nuclear localized?	Lalanne *et al.,* 2004b
unknown transmembrane protein	plasma membrane, 6 TM domains, potential protein-protein interaction, and a single WD-40 motif.	Lalanne *et al.,* 2004b
calcium dependent protein kinase	signalling	Lalanne *et al.*, 2004b
auxin-indiced protein (AIR9)	function unknown	Lalanne *et al.*, 2004b
fructokinase-like protein	metabolism	Lalanne *et al.*, 2004b
Clathrin adapter medium chain, MU1B (Mus musculus)	membrane trafficking	Johnson *et al.,* 2004
26s proteasome regulatory subunit S3	protein synthesis or degradation	Johnson *et al.,* 2004
Sucrose transporter, SUC1; 60S ribosomal protein L10	sucrose transport (cell wall biosynthesis, energy)	Johnson *et al.,* 2004
Ribophorin II (Homo sapiens)	membrane trafficking	Johnson *et al.,* 2004
Unknown	unknown	Johnson *et al.,* 2004
SABRE-like protein	tip growth	Procissi *et al.,* 2003
Vps52p/SAC2 protein (yeast)	tip growth, retrograde golgi vesicle transport	Lobstein *et al.,* 2004
Mago nashi (Drosophila melanogaster)	gene expression?	Johnson *et al.,* 2004
Unknown	unknown	Johnson *et al.,* 2004
Ubiquitin extension protein 1 (UBQ1)/60S ribosomal protein L40	gene expression?	Johnson *et al.,* 2004
Alcohol dehydrogenase-like protein	function in metabolism?	Lalanne *et al.*, 2004b
GTP-binding typA-related protein	function unknown	Lalanne *et al.*, 2004b
putative protein	cytosolic, putative function unknown	Lalanne *et al.*, 2004b
Unknown; Mitochondrial ATP synthase d chain (Ipomoea batatas)	energy production	Johnson *et al.,* 2004
VANGUARD1, pectin methyl esterase	cell wall modification	Jiang *et al.,* 2005

Outline of the thesis

In this study, I aim to identify unknown pollen-expressed genes involved in the progamic phase of sexual plant reproduction. To do so, I used a genetic approach with *Arabidopsis* as model system, and I characterized a male gametophytic mutant identified by its segregation distortion phenotype, in which pollen development was unaffected. As most studies on pollen tube growth were performed previously on bicellular tobacco, lily or poppy pollen, which might be different from that of the tricellular *Arabidopsis* pollen, I studied pollen tubes of *in vitro* germinated *Arabidopsis* pollen at the ultrastructural level, using light and electron microscopy (Chapter 2). In Chapter 3, a detailed analysis of the defect in male transmission of the male gametophytic mutant line TJ995, the T-DNA insertion pattern and the isolation of the mutated gene encoding a putative family 1 glycosyl transferase are presented. Phenotypic analysis of the mutant is described in Chapter 4 and revealed the *bursting pollen (bup)* phenotype, indicative of a pollen germination defect. Gene expression analysis of *BUP*, as well as the expression of the closely related gene *BUP-like (BPL)* is presented in Chapter 5. Finally, protein localization studies were performed to analyse whether BUP was localized within the Golgi bodies, like most type II membrane protein glycosyl transferases (Chapter 6). A discussion on the function of BUP on pollen tube growth is presented in Chapter 7.

Chapter 2

Growth and cellular organization of *Arabidopsis* pollen tubes *in vitro*

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Abstract

Tricellular pollen tubes of *Arabidopsis thaliana* were cultured *in vitro* on solid media and studied with respect to growth, cellular organization and ultrastructure, cytoskeletal organization, organelle movement, deposition and structure of the wall and the occurrence of coated pits, all elements assumed to be relevant for tip growth. For our ultrastructural studies we used freeze fixation and freeze substitution. Although *Arabidopsis* pollen tubes are broadly similar to those of bicellular species such as *Nicotiana tabacum* and *Lilium* spec. and *in vivo* grown pollen tubes of *Arabidopsis*, some differences occurred. The density of the equally distributed, relatively small (85 nm) secretory vesicles in the tip is low (five/μm^2). In between the secretory vesicles of the tip, membranous material, possibly smooth endoplasmic reticulum, fragments of rough endoplasmic reticulum and loose ribosomes are present. The wall in the tip is not amorphous but layered and a secondary wall is formed already in the flanks of the tip. The general pattern of organelle motion is reverse fountain-like, but individual organelles move in distinct lanes at speeds of upto 2 µm/s, and about half of the organelle population shows a moderate velocity or Brownian movement. These properties are discussed in relation to the low growth rate (10 µm/h) of *Arabidopsis* pollen grown *in vitro*. The two similar sperm cells are closely attached and are always found near the vegetative nucleus. No surrounding wall and no cytoskeletal elements were obvious in the sperm cells. The preferential location of the mitochondria at the wall and the large (up to 400 nm) coated pits are unique for angiosperm pollen tubes. The size of the coated pits may allow not only membrane retrieval but also pinocytosis.

Introduction

Pollen tubes have attracted much interest not only because of their role in fertilization, but also as a model system for polar cell growth. Present views on pollen tube biology derive mainly from species with bicellular pollen, in which the sperm cells are formed after germination, such as *Lilium* and *Nicotiana tabacum*. Such pollen tubes are capable of fast autonomous growth *in vitro* as a result of the extensive secretion of dictyosome- (or Golgi-) derived vesicles (secretory vesicles) at the tip of the cell. Characteristics of such pollen tubes are a tip-focused cytoplasmic calcium gradient, a zonal distribution of organelles, an axially organized cytoskeleton, a reverse fountain-like cytoplasmic streaming pattern, and a broad zone with coated pits behind the tip. The primary wall secreted at the tip consists largely of pectin, whereas the secondary wall that is deposited behind the growing tip consists mainly of callose (Pierson and Cresti, 1992; Derksen *et al.,* 1995a; Taylor and Hepler, 1997; Malhó, 1998b; de Win *et al.,* 1999; de Graaf *et al.,* 2001; Hepler *et al.,* 2001).

Tricellular pollen, with two sperm cells at pollen maturation, has attracted less attention than bicellular pollen due to difficulties in germination and pollen tube growth *in vitro.* Apart from a few reports on various aspects of growth *in vivo* (e.g., Kandasamy *et al.,* 1994), little information is available on the cellular organization of their pollen tubes, including those of *Arabidopsis thaliana*, the plant of choice for genetic and molecular studies (The *Arabidopsis* Genome Initiative, 2000). A recent study on *Arabidopsis* pollen tubes by Lennon and Lord (2000) focused on general cellular organization of pollen tubes grown *in vivo,* and in particular on wall secretion and composition. Studies that address genetic anomalies in male gametophytes clearly need more detailed data on wild type *Arabidopsis* pollen tubes as a reference, without interference from uncontrolled external factors. Here, we present the first study of cellular organization and ultrastructure of tricellular pollen tubes grown *in vitro*. Special attention was paid to cytoskeletal organization, organelle movement, the pollen tube wall and the occurrence of coated pits, all elements assumed to be relevant for tip growth. From our results we conclude that some differences found between *Arabidopsis* pollen tubes grown *in vivo* and bicellular pollen tubes (Lennon and Lord, 2000) are to be attributed to growth *in vivo* versus growth *in vitro.*

Material and methods

Plant material

Pollen from *A. thaliana* ecotype Landsberg erecta was collected by tapping open flowers on small pieces (3 mm^2 for electron microscopy and about 5 mm^2 for light microscopy) of Visking dialysis membrane (Serva, Germany). The membranes were boiled for 6 h in water and soaked in liquid medium before use. The membranes were placed on semi-solid medium (Hodgkin, 1983) consisting of 0.01% H_3BO_3, 0.7% Bacto-Agar (Difco, Detroit, Mich.), 0.07% $CaCl_2 \cdot 2H_2O$, 3.0% polyethylene glycol 4,000 (Duchefa, The Netherlands) and 20% sucrose (Mallinkrodt Baker, The Netherlands), pH 7. In some experiments, excised *Arabidopsis* pistils were added to the membranes. Pollen was germinated and grown for 6–24 h (overnight) in the dark at 27°C. All chemicals were from Merck (Germany), unless stated otherwise.

Light microscopy

Analysis of growth on semi-solid medium was carried out by direct or time lapse observation of material cultured in sealed Petri dishes using an Axiovert inverted microscope (Zeiss, Germany). For organelle movement analysis, pollen tubes were transferred to a drop of liquid medium on a microscope slide, covered with a coverslip and immediately studied. Observations could be carried out for 25 min without noticeable effect on organelle motion. Observations were made with the Axiovert microscope equipped with optovar magnification lenses and connected to a Chalnicon C2400 camera unit (Hamamatsu Photonics, Japan), stored on analogue tapes and evaluated by observation on screen. Actin filaments were labelled with Alexa(488)-phalloidin (Molecular Probes, Eugene, Ore.), at about 13 µM final concentration after fixation for 4 h in a mixture of 3% freshly dissolved paraformaldehyde in 20% sucrose, 100 mM potassium phosphate buffer (pH 7.2) and 10 mM EGTA, followed by extensive rinsing in 5% DMSO, 17% sucrose, 5 mM EGTA and 100 mM potassium phosphate buffer (pH 7.2). Nuclei were labelled with 1 µM 4,6-diamino-2-phenylindole (DAPI; AppliChem) as fluorescent probe for DNA (Sanders *et al.,* 1990). Microtubules were visualized according to standard procedures (Derksen *et al.,* 1985) with some adaptations. Pollen tubes were fixed for 1 h in 50 mM potassium phosphate buffer (PPB, pH 8), 4% (w/v) paraformaldehyde, 5 mM EGTA, 5% DMSO, then washed in PPB, subsequently immobilized with a thin layer of 1% gelatin (w/v), 1% agarose, 7% glucose and digested at 37°C for 1 h with a mix of 0.5% (w/v) helicase, 0.5% (w/v) cellulase, 0.5% (w/v) pectolyase in PPB. After rinsing with PPB, pollen tubes were incubated for 30 min with 1% Triton X-100 in PPB, rinsed twice in 0.02 M sodium phosphate buffer pH 8, and incubated overnight at room temperature with 5% (v/v) rat anti-tubulin (MAS 077b, Harlan Sera-Lab, Loughborough, England), in 0.02 M sodium phosphate buffer pH 8, 0.3 M NaCl, 0.1% (w/v) sodium azide, 0.05% Triton X-100, and 4.5 mg/ml BSA. After six rinses with 0.02 M sodium phosphate buffer pH 8, pollen tubes were treated with 0.5% (v/v) FITC-labelled secondary antibody (labelled goat anti-rat IgG Alexa fluor 488, A-11006; Molecular Probes Europe) at 37°C (Procissi *et al.,* 2003). Pollen tubes were rinsed six times in PPB, and then mounted in antifade (Citifluor, Agar Scientific, Stansted, UK). Images were captured with a Leica TCS-NT CLSM (Leica Microsystems, Germany) equipped with an argon/krypton laser (Omnichrome, Chino, Calif.). Callose was stained with decolorized Aniline Blue (Polysciences, Warrington, Pa.) and crystalline wall material with Calcofluor White (American Cyanamid, Parsippany, N.J.). Unless stated otherwise, micrographs were taken with a conventional fluorescence microscope (Leitz Orthoplan/Ortholux combination) equipped with the appropriate illumination and filters for excitation and emission.

Transmission electron microscopy

The transmission electron microscopy procedure was essentially as described previously (Derksen *et al.,* 1995b). In short, membranes with pollen tubes grown for about 3 h were plunged into liquid propane 3.5 (Air Liquide, Belgium; –175°C), transferred into liquid nitrogen and freeze substituted in a Reichert-Jung (Austria) freeze-substitution apparatus. After substitution at –90°C for 36 h in 1% OsO_4 and 0.01% uranyl acetate in acetone, the temperature was slowly raised (4°C/h) to room temperature.

Membranes with pollen tubes were embedded in Spurr's resin (Agar Scientific) and mounted on Sylon-CT-coated (Supelco, Bellefonte, Pa.) slides. The dialysis membrane was removed during the trimming process. Longitudinal sections were made with a MT5000 microtome (Sorvall, Newton, Conn.), post-stained with 0.3% uranyl acetate for 3 min, and examined in a 100CX II transmission electron microscope (Jeol, Tokyo, Japan). All observations were carried out on longitudinal median sections of pollen tubes from three independent experiments. Dimensions of organelles were estimated from sections of at least two tubes from three independent experiments. Secretory vesicle diameters were estimated from the entire clear zone area. Dictyosome measurements were carried out on at least five dictyosomes per section.

Results

Growth and organelle movement

The germination percentage was 50% on average. Addition of excised *Arabidopsis* pistils resulted in a relatively small increase in this percentage. Although the growth rates varied, most tubes reached a length of about 250 μm after 24 h. The pollen tubes were straight, with a diameter between 5 and 7 μm. During growth, swelling of the tube occurred in periods that coincided with recovery of growth after a temporary slow down or arrest of growth. The pattern of organelle movement was mainly reverse fountain-like. Due to the small size and strong curvature of the wall, only larger organelles such as mitochondria and dictyosomes may be seen. Individual secretory vesicles could not be identified and followed, mainly due to their small size. Approximately half of the visible organelles exhibited vigorous movement, up to 2 μm/s in distinct lanes. The other half showed little, or only Brownian, movements. A clear zone was present in the tip (data not shown).

Cytoplasmic organization

Figure 1 shows a longitudinal, median, section through the entire germinated pollen grain and the tube. Inside the grain, the exine and intine layers and the large central vacuole can be clearly recognized. The two sperm cells are present near the pollen grain. Extended vacuoles can be seen throughout the tube until about 15 μm from the tip. Darkly stained mitochondria are present throughout the cytoplasm, but mostly along the periphery of the cell. Figure 2 shows a more precise median section through the growing tip. Secretory vesicles are present in the tip, and larger organelles are occasionally found. Occasionally, fragments of rough endoplasmic reticulum (RER) occur. Behind the dome, dictyosomes, small vacuoles and sheets of RER can be seen. A little further from the tip, many mitochondria are visible. Oblique sections show that mitochondria are preferentially located close to the tube wall (not shown). Larger organelles, such as multi-vesicular bodies, preplastids and lipid bodies are found throughout the cytoplasm, but at a very low frequency (1–5 per section in figure 1). In the clear zone at the tip many small secretory vesicles, with a maximum diameter of 85 nm, are observed. However, their density is relatively low, about five/μm^2. As shown in figure 3, secretory vesicles are interspersed with membrane material, probably smooth endoplasmic reticulum (SER) and electron dense ribosomes.

Wall deposition and wall structure

The dictyosomes, or Golgi bodies, always showed the typical structure with cis-media- and trans-cisternae and an extensive trans-Golgi network (TGN) (not shown) bordered with electron dense, small and smooth coated vesicles. Figure 4 shows a dictyosome near to the tip. The small, smooth CV with a diameter of about

35 nm border the cisternae and the TGN (figures 4 and 5). Clathrin-coated vesicles could not be found. At the TGN larger, relatively less electron dense, vesicles can be observed that eventually mature into secretory vesicles (figure 5). In contrast, the secretory vesicles found in the cytoplasm were rather electron translucent, though their cortex always remained electron dense (figure 5). Secretory vesicles captured at fusion with the plasma membrane and the wall of the tip have a more electron dense aspect, similar to that of the primary wall (figure 6). A few coated pits with a variable diameter, between 50 and 400 nm, are always found along the apical plasma membrane, especially at the flank of the dome (figure 7). Coated vesicles are only seldom seen at the cell membrane of the tip (not shown). The wall of the very tip is electron dense, clearly striated with a fluffy outer border (figure 6). Already in the dome of the tip an electron translucent layer representing the secondary wall can be discerned (figure 7). Immediately behind the dome, the primary wall gradually becomes thinner, while the secondary wall becomes thicker (figure 8). At some distance from the tip, usually not more than 50 µm, the secondary wall seems completed, while the primary wall has disappeared almost entirely (figure 9). Both Calcofluor White staining for crystalline wall components (figure 10) and Aniline Blue staining for callose (figure 11) show a clear lining of the tube walls, with the difference that Calcofluor White stains the tip but Aniline Blue does not. Aniline Blue staining shows only a slight increase in fluorescence intensity from the tip to the base of the tube, indicating only a small increase in wall deposition during growth. Aniline Blue also stains callose plugs; between two and five plugs are present in the pollen tube after 24 h.

Sperm cells and vegetative nucleus

The two sperm cells in the tube are spherical, with a maximum diameter of about 2 µm, and they always appear closely attached. Usually, they are behind the vegetative nucleus. Although sperm cells and vegetative nucleus often migrate close to each other, no clear connection between sperm cells and vegetative nucleus was observed in our electron microscopic preparations. The vegetative nucleus is always euchromatic with numerous nuclear pores (figure 12). The nuclei of the sperm cells also appear euchromatic (figure 13) but nuclear pores are seldom seen. In the cytoplasm of the sperm cells, no microtubules or actin bundles could be observed, but a few mitochondria, small dictyosomes and sometimes other organelles were always present. We did not find obvious differences between the two sperm cells. No wall was apparent between the membranes of the vegetative nucleus and sperm cells.

Cytoskeleton

With Alexa-phalloidin as a probe, more or less randomly organized actin filaments can be detected in the tip. In the tube, the actin filaments appear

organized in a more linear fashion with increasing distance from the tip (figure 14). Sections showed the presence of typical bundled actin filaments behind the growing tip (figure 15). Microtubules in the tube were detected by immunohistochemistry. They seemed to be largely absent from the tip. In the tube they were cortical and mainly axially oriented (figure 16). These observations are consistent with those obtained by electron microscopy. A few short and randomly organized microtubules occur in the tip (not shown). Directly behind the tip, numerous, more or less axially oriented, microtubules can be seen in oblique sections (figure 17). Electron microscopic preparations show that, at some distance from the tip, microtubules are cortical, relatively long and axially oriented (figure 18). Thus, *Arabidopsis* pollen tubes posses a typical axially organized cytoskeleton with mainly cortical microtubules. Both actin filaments and microtubules are present in and near the tip, but in a less regular fashion.

Next page:

Figure 1: Longitudinal, median section through a pollen grain and the emerged tube after 8 h of culture. The exine (E) and intine (I) walls and the large central vacuole of the pollen grain are clearly visible. The secretory zone (SZ) in the tip of the tube is essentially devoid of larger organelles. Darkly stained mitochondria appear directly behind the tip and can be seen throughout the tube. At about 10 µm behind the tip, indicated by the dotted line, long extended vacuoles can be observed. Their extension reflects the axial orientation of organelle movements. In the basis of the tube the two closely associated sperm cells (SC) can be seen. Bar 5 µm

Figure 2: Precise longitudinal, median section through the tip. The tip is devoid of larger organelles. Secretory vesicles (SV, arrows) are present throughout the tip. Interspersed with the SV are large amounts of membranous material, mostly smooth endoplasmic reticulum (SER), but also rough endoplasmic reticulum (RER). Behind the tip, darkly stained mitochondria (M), dictyosomes (D) and RER (pointers) are visible. Bar 2 µm

Figure 3: High magnification of part of the tip shown in figure 1. In addition to secretory vesicles (arrow), electron dense particles, ribosomes and membrane material, possibly SER (pointers) are present. Bar 0.1 µm

Figure 4: Planar section through a cisterna of a dictyosome. The smooth coated vesicles (arrow) are clearly visible. The cisterna is surrounded by a clear zone (asterisk). Bar 0.2 µm

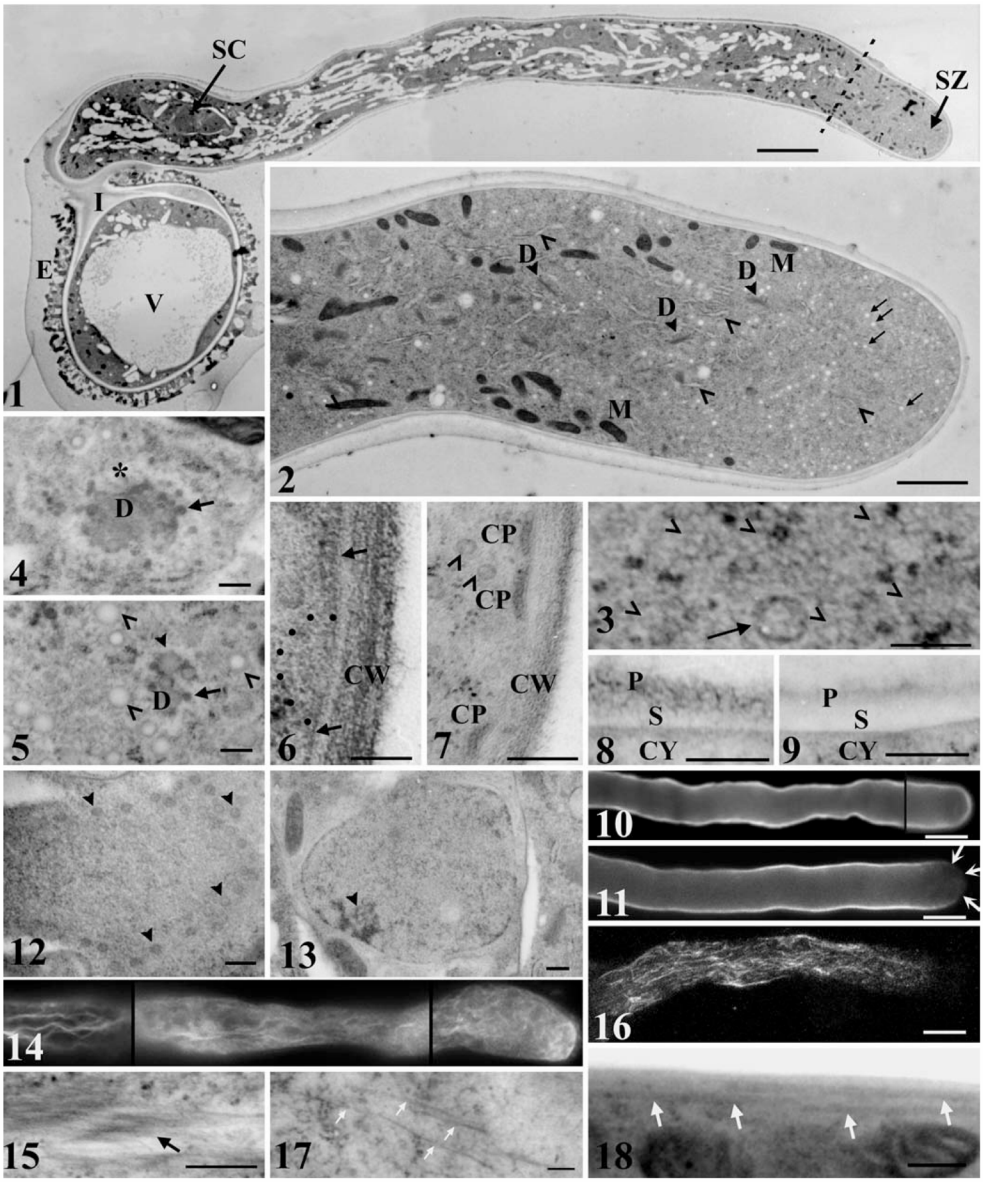

Figure 5: Section through part of the trans-Golgi-network (TGN) of a dictyosome (D). Smooth coated vesicles (arrow) are visible together with larger and more darkly stained budding vesicles (arrowheads) and mature secretory vesicles (pointers). Note the dark cortex and electron translucent core of the secretory vesicles. Bar 0.2 µm

Figure 6: Transverse section through the cell wall in the tip. The wall is layered and seems fluffy at the outside. At the inside, the cell membrane with its dark lining is visible (arrows). A fused secretory vesicle with the same electron density as the wall is indicated (dotted curve). Bar 0.2 µm

Previous page:
Figure 7: Transverse section through the cell wall (CW) at the flank of the dome. The wall appears fluffy at the outside. At the membrane, typical coated pits (CP) are present. Secretory vesicles (pointers) with a dark cortex are visible. The dark points represent ribosomes. Bar 0.2 µm

Figure 8: Transverse section through the wall behind the tip. The darkly stained primary wall (P) is thinner than that in the tip, but its fluffy aspect is more pronounced and layers are no longer visible. The electron translucent, secondary and callosic wall (S) is prominent. Cytoplasm (CY). Bar 0.2 µm

Figure 9: Transverse section through the wall at about 20 µm behind the tip. The darkly stained primary wall (P) has almost disappeared. A thick secondary, callosic wall (S) can be seen. Cytoplasm (CY). Bar 0.2 µm

Figure 10: Pollen tube stained with Calcofluor White. No obvious differences in staining intensity are apparent between the tip region (right part of panel), and the basis of the tube (left part of panel). Bar 5 µm

Figure 11: Pollen tube stained with Aniline Blue. The very tip (arrows) is not stained. The staining becomes apparent in the flanks of the dome. Behind the dome, no obvious differences in staining intensity are apparent. Bar 5 µm

Figure 12: Oblique section through the vegetative nucleus (VN) showing numerous typical nuclear pores (arrowheads). No heterochromatin is visible. Bar 0.2 µm

Figure 13: Section through one of the sperm cell nuclei (SN). No nuclear pores can be seen in this section. Only a little heterochromatin (arrowhead) is visible. Bar 0.2 µm

Figure 14: Actin filaments stained by fluorochrome-coupled Phalloidin. At the tip (right part of panel), no clearly oriented filaments can be observed. At a distance of about 20 µm from the tip, actin filaments are present predominantly in a longitudinal fashion (middle part of panel). Typical, longitudinal actin filaments are present at the basis of the tube (left part of panel). Bar 5 µm

Figure 15: Section through a bundle of microfilaments in the middle part of the tube showing the typical fibres of the individual filaments (arrow). Bar 0.2 µm

Figure 16: Immunohistochemically detected microtubules. The image is a projection of 16 optical sections made with the confocal laser scanning microscope. Hardly any staining is present in the tip. At about 5 µm from the tip, the first clear microtubules can be detected. Further from the tip microtubules are more longitudinally and cortically organized. Bar 5 µm

Figure 17: Oblique section behind the tip at a distance of about 20 µm behind the tip showing nearly axially oriented, cortical microtubules (arrows). Bar 0.2 µm

Figure 18: Longitudinal section through the tube at a distance of about 75 µm behind the tip showing the cortical, axially oriented microtubules (arrows). Bar 0.2 µm

Discussion

Arabidopsis and bicellular pollen tubes *in vitro*

The cellular organization of *Arabidopsis* pollen tubes is broadly similar to that of bicellular species (Cresti *et al.,* 1977; Pierson and Cresti, 1992; Derksen *et al.,* 1995a; Taylor and Hepler, 1997; Malhó, 1998b; de Win *et al.,* 1999; de Graaf *et al.,* 2001; Hepler *et al.,* 2001), showing (1) a growing tip where large organelles are absent, (2) wall secretion in the tip by a single type of secretory vesicles, (3) the presence of coated pits and coated vesicles at the tip, (4) a layered primary cell wall that disappears after secretion of the secondary, callosic wall, (5) an axially organized cytoskeleton with more irregularly distributed elements near the tip, and (6) a reverse fountain-like cytoplasmic streaming, yet with highly individual organelle movement in distinct lanes. Differences from bicellular pollen, such as the low density of the relatively small secretory vesicles in the tip, the preferential location of mitochondria at the wall and the moderate velocity or Brownian movement of part of the organelle population may be related to slow growth *(Arabidopsis in vitro*: 10 µm/h) as suggested for *Pinus sylvestris* (1 µm/h; de Win *et al.,* 1996). In comparison: the growth rate for lily and tobacco is about 10 µm/min (Derksen *et al.,* 1995a). The presence of actin in the tip indicates a role in tip growth (Fu *et al.,* 2001), but the reliability of staining patterns in the tip of fixed pollen tubes is disputed and final conclusions therefore cannot be drawn (Lancelle *et al.,* 1987; Derksen *et al.,* 1995a; Fu *et al.,* 2001). The absence of an amorphous wall at the very tip, and the closeness of the coated pits and secondary wall to the tip, may yet again relate to slow growth. These different properties are obviously no impediment to tip growth.

Arabidopsis pollen tubes *in vitro* and *in vivo*

Both similarities and differences between *Arabidopsis* and bicellular pollen tubes grown *in vitro* (this study) also apply to *in vivo* grown *Arabidopsis* pollen tubes (Lennon and Lord, 2000). As expected, the growth rate *in vitro*, 250 µm in 24h, is much lower than that *in vivo*, here similar lengths are obtained after only 3 h, although the spatial properties of the tubes are about the same. Our observations on the cortical location of mitochondria and the absence of a distinct organization of nuclear and organelle zone differ from those on *in vivo* growing pollen tubes. Stacks of RER or large amounts of osmiophilic globules were not observed *in vitro*. The occurrence of plasmatubules *in vivo* but their absence *in vitro* is consistent with observations in tobacco (Kandasamy *et al.,* 1988) and lily (Roy *et al.,* 1997), thus indicating a significant difference between growth *in vitro* and *in vivo*. The dictyosomes and secretory vesicles are very similar in structure *in vitro* and *in vivo*. The small smooth coated vesicles bordering the dictyosomes were also observed in tobacco (Derksen *et al.,* 1995b). They were thought to

concern intra-Golgi transport, but may also be involved in other shuttling routes. The staining pattern of the secretory vesicles *in vitro* – a light core and dark cortex – appears reversed *in vivo*. In our hands such differences occurred also in other tissues and other types of pollen tubes depending on as yet undetected minor variations in the substitution procedure.

In contrast to the situation *in vivo*, clusters of secretory vesicles are absent *in vitro* but large amounts of membranous material, probably SER, some RER, and apparently loose ribosomes do occur in the tip. This arrangement resembles that seen in slow growing pine pollen tubes (de Win *et al.,* 1996). The extreme differentiation of the tip, as in lily (Lancelle *et al.,* 1997) and, albeit to a lesser extent, in tobacco and petunia (Derksen *et al.,* 1995b, 1999) apparently elates to speed of growth. In general, coated pits in plant cells do not become larger than 170 nm, the minimum size for coated vesicles formation yet considered too small for pinocytosis (Newcomb, 1980). The coated pits in the *in vitro* grown pollen tubes of *Arabidopsis* are much larger (50–400 nm) than those in other plant cells including lily, tobacco and pine pollen tubes grown *in vitro* (Pierson *et al.,* 1986; Derksen *et al.,* 1995a; de Win *et al.,* 1996). Therefore, though not yet reported *in vivo*, coated pits in *Arabidopsis* pollen tubes could be involved in pinocytosis in addition to membrane retrieval.

The high speed of growth *in vivo* may relate to an efficient supply of nutrients and to so far unknown growth factors from the female sporophytic tissue. A specific intense contact between pollen tube and the female sporophytic tissues *in vivo* is demonstrated by the presence of plasmatubules in pollen tubes *in vivo* only. In addition, the large coated pits indicate the presence of a communication pathway that cannot be functional *in vitro*.

Vegetative nucleus and sperm cells

The vegetative nuclei are highly euchromatic with numerous nuclear pores, which relates to a high level of activity (Wagner *et al.,* 1990). *Arabidopsis* sperm cells are small with little cytoplasm and occur in pairs. No differentiation such as occurs in *Plumbago* (Russell and Cass, 1981) was apparent. The nuclei are euchromatic but show few nuclear pores, which indicates a low level of general activity (Wagner *et al.,* 1990). Unlike *in vivo*, no obvious wall was found around sperm cells *in vitro* (Lennon and Lord, 2000). No obvious cytoskeletal elements could be observed in sperm cells of *in vitro* cultured pollen tubes. Microtubules are clearly present in electron microscopic preparations of the sperm cells from *Hyacinthus orientalis* (Del Casino *et al.,* 1992), *Spinacea oleracea*. (Theunis *et al.,* 1991), *N. tabacum* (Yu and Russell, 1994) and *Brassica oleracea* (Cresti *et al.,* 1990), but not in sperm cells from *Plumbago zeylanica* (Russell and Cass, 1981). Their apparent absence or rarity in our electron microscopic preparations is unlikely

to be biased, as we used freeze fixation and freeze substitution (Lancelle *et al.,* 1987).

Conclusion

Arabidopsis pollen tubes cultured *in vitro* are broadly similar to pollen tubes grown *in vivo*, but also to *in vitro* cultured bicellular pollen tubes. Some of the dissimilarities probably relate to differences in growth speed. Plasmatubules and the secretory vesicles clusters in the tip seem unique to pollen tubes grown *in vivo* (Lennon and Lord, 2000); they do not occur *in vitro*. The large size of the coated pits (400 nm) is unique for plant cells and could allow pinocytosis.

Chapter 3

Genetic and molecular analysis of a T-DNA insertional male gametophytic mutant

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Abstract

In order to investigate male gametophytically expressed genes involved in pollen tube growth and pollen tube guidance, the Tom Jack *Arabidopsis* T-DNA mutant population was screened by segregation distortion analysis. This led to the identification of line TJ995 with a T-DNA segregation distortion of 1:1 resistance to sensitive seedlings. This segregation distortion was shown to be caused by a severely reduced male T-DNA transmission efficiency (TE=1%), while female TE was normal, indicating a male gametophyte-specific defect. Southern blot analysis showed that line TJ995 contains five or six T-DNA copies, organized in tandem and inverted repeats at three different insertion sites. Although we did not succeed in recovering all insertion sites by inverse PCR and TAIL-PCR, mainly due to the complex integration pattern and the presence of backbone plasmid DNA, one of the T-DNA insertions was found to be located in gene At5g04480. Reverse genetics segregation distortion analysis of an independent T-DNA insertional mutant of gene At5g04480 also showed a male-specific defect in T-DNA transmission. In addition, a molecular complementation test with the wild-type gene fully restored the segregation distortion phenotype in both line TJ995 and in this SALK mutant, proving that the defect in male T-DNA transmission is caused by the mutation of gene At5g04480 alone. Protein domain analysis suggests that the single transmembrane gene product of At5g04480 is a group 1 glycosyl transferase with a putative role in the synthesis of glycoproteins or cell wall polysaccharides. The predicted protein shows homology with two rice proteins and one other *Arabidopsis* protein, suggesting that this protein is conserved through evolution and therefore might also be important for pollen development or pollen tube growth in other flowering plants.

Introduction

In flowering plants, the gametophytic phase of the life cycle starts with the development of the gametophytes, which are produced in the reproductive organs of the flowers. The development of the pollen or male gametophyte and the embryo sac or female gametophyte is regulated by sporophytic genes (expressed in the diploid anther and pistil) and by gametophytic genes (expressed in the developing haploid gametophytes). Some of these genes also play an important role during pollen-pistil interaction, i.e. pollen germination on the stigma, directional pollen tube growth within the pistil, delivery of the sperm cells and finally the fertilization events.

In order to investigate genes involved in sexual plant reproduction, mutant populations have been screened for mutations affecting this process. Plants with a lethal gametophytic mutation can only transmit the mutation to the offspring through the unaffected gametophytes, and therefore can only be recovered as heterozygotes. A male gametophytic mutation thus will affect half of the pollen resulting in fully self-fertile plants, due to the large excess of wild-type pollen relative to the number of ovules available for fertilization. The absence of a clear sterility phenotype makes it difficult to identify these mutants in large mutant populations.

In T-DNA insertional *Arabidopsis* mutant populations the T-DNA 'tags' the mutation, facilitating the isolation of the mutated gene. The segregation of T-DNA-induced mutations in the progeny can be followed using the selection marker of the T-DNA, for example the NPTII gene that confers resistance to the antibiotic Kanamycin on selection plates. Normally, a single T-DNA insertion would segregate in a Mendelian manner, resulting in 3:1 Kanamycin resistant to sensitive seedlings upon self fertilization of a hemizygous plant. As gametophytic mutants fail to transmit the T-DNA linked to the mutation to the offspring through the affected gamete, the defect in T-DNA transmission will instead result in a segregation of 1:1 resistant to sensitive seedlings (Feldmann *et al.*, 1997; Howden *et al.,* 1998). Using this assay of segregation distortion, numerous male gametophytic mutants, female gametophytic mutants and plants carrying a mutation affecting the function of both gametophytes have been isolated (Howden *et al.,* 1998; Grini *et al.,* 1999; Bonhomme *et al.,* 1998a, 1998b; Christensen *et al.,* 1998; Procissi *et al.,* 2001; Lalanne *et al.*, 2004; Johnson *et al.*, 2004).

T-DNA insertional mutagenesis is widely used to generate mutant populations that can be screened for a mutant phenotype (forward genetics) or for a mutated gene (reverse genetics). However, in only 35-40% of the T-DNA insertional mutants the mutant phenotype is actually linked to a T-DNA insertion. The other mutants generally result from deletions or T-DNA footprints, probably due to abortive integration events (Azpiroz and Feldmann, 1997). Also when a T-DNA is

integrated successfully, many modifications to the clear single T-DNA insertion can be found. The T-DNA can be truncated and T-DNA mutants often contain multiple T-DNA copies, which are organized in direct or inverted repeats or are inserted at different loci in the genome (Gheysen *et al.,* 1991). Furthermore, T-DNA transformation can lead to major chromosomal rearrangements by duplication and translocation of chromosomal regions (Castle *et al.,* 1993; Nacry *et al.,* 1998; Tax and Vernon, 2001), and finally the integration of vector backbone sequences might complicate the identification of adjacent plant DNA (Martineau *et al.,* 1994; Wenck *et al.,* 1997; De Buck *et al.,* 2000). Since all these modifications can complicate the molecular analysis of the mutant gene, detailed genetic analysis of all T-DNA insertion sites of a T-DNA mutant is needed to exclude the possibility that other mutations within the genome are affecting the mutant phenotype.

In this chapter the identification of a male gametophytic mutant in a screen for segregation distortion is presented. Genetic and molecular analyses were performed that have led to the identification of the affected At5g04480 gene. The essential role of this gene in the progamic phase of reproduction was confirmed by the segregation analysis of an independent mutant allele and by molecular complementation. Protein structure analysis showed similarity with glycosyl transferases belonging to the glycosyl transferase family 1, suggesting a role in the synthesis of cell wall components or the glycosylation of proteins.

Material and methods

Plant material

Seeds of *Arabidopsis thaliana* L. ecotype Col-O and Col-6 (Col-0 with *glabra1* mutation, ABRC stock number CS8155) were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC). *Arabidopsis* plants were grown in a 3:1 mixture of soil and vermiculite and kept under greenhouse conditions with a 16 h photoperiod, at 22°C and ambient relative humidity. The line TJ995 was isolated from a set of pooled Tom Jack enhancer trap T-DNA lines (ABRC stock numbers CS31086 and CS31087) in the Col-6 background, transformed by vacuum infiltration with the binary vector pD991 (Campisi *et al.,* 1999). The plant used for transformation is referred to as the T0 generation, T1 are the primary transformants, and T2 and T3 are progeny of respectively the T1 and T2 generation after self-fertilization. SALK line 033889 was ordered from NASC. Insertion mutant information was obtained from the SIGNAL website (http:// signal.salk.edu). The T-DNA insertion sites of TJ995 and SALK_033889 were confirmed by PCR as described in the section *'Cloning of sequences flanking the T-DNA insertions '*.

TJ995 and SALK seeds were surface sterilised by incubation for 2 minutes in 96% ethanol, followed by 5 minutes incubation in a solution of 40% bleach and 0.1% Tween-20. The seeds were washed at least 4 times with sterile dH_20, and pipetted onto selection plates in 0.1% select agar. Alternatively, the seeds were sterilized in a drop of 95% ethanol. Ethanol was allowed to evaporate overnight. Sterilized seeds were plated onto Kanamycin (50 mg/l)-supplemented medium (2.2 g/l MS salts, 0.5 g/l MES, pH 5.7 and 7 g/l select agar, pH 5.8). After 3 days at 4°C, plates were incubated under 12h photoperiod at 21°C. Antibiotic resistance phenotypes were scored after one to two weeks and resistant seedlings were transferred to pots containing a 3:1 compost:sand mix and grown under greenhouse conditions with supplemental lighting (16 h of light at 22°C).

Genetic transmission analyses

Transmission of mutations through the male and female gametes was determined by carrying out reciprocal testcrosses of hemizygous mutants and wild-type (Col-0, Col-6 or male-sterile *ms1-1* ecotype Lansberg erecta) plants. Male and female transmission efficiencies (TEmale and TEfemale) were calculated according to Howden *et al.* (1998). The expected number of gametes carrying the trait is taken to equal the number of gametes lacking it, assuming random segregation during meiosis and the absence of post- meiotic selection; therefore, TE (%)=(observed R/observed S) x 100.

Genomic DNA isolation

Genomic DNA was isolated from *Arabidopsis* rosette leaves. Approximately 2 g of leaves frozen in liquid nitrogen were homogenized in a 50 ml Falcon tube by vortexing with a marble. 20 ml freshly prepared cold extraction buffer (0.1 M Tris-base, 5mM EDTA, 0.35 M Sorbitol and 20 mM $Na_2S_2O_5$, pH 7.5) was added and the plant homogenates were kept on ice for 30 min. The samples were centrifuged at full speed at 4°C for 20 min in a table centrifuge. The supernatant was discarded and the pellet was resuspended in 1.25 ml of extraction buffer. Additionally, 1.75 ml Nuclear-Lysis-buffer (0.2 M Tris-base, 0.05 M EDTA, 2.0 M NaCl and 2% CTAB) and 0.6 ml of 5% (w/v) Sarkosyl were added and the tubes were inverted 5-10 times at room temperature, followed by incubation for 20 min at 65°C. The mixtures were extracted several times with an equal volume of chloroform/isoamylalcohol (24:1) at room temperature and the DNA was precipitated with one volume of cold isopropanol and resuspended in 100 µl TE. Finally, the precipitation step was repeated with 0.1 volume of 3.0 M NaAc and 2.2 volumes of EtOH to eliminate remnants of the CTAB which disturbs the quantitative analysis of the genomic DNA.

Cloning of sequences flanking the T-DNA insertions

Genomic sequences flanking the right and left T-DNA borders were amplified with thermal asymmetric interlaced (TAIL) PCR or Inverse PCR. TAIL-PCR was performed according to Liu *et al.*

(1995) with minor modifications. T-DNA-flanking sequences were amplified in three rounds of PCR using one of the three degenerate TAIL-PCR primers AD1: 5'-NTCGA(G/C)T(A/T)T(G/C)G(A/T)GTT-3', AD2: 5'-NGTCGA(G/C)(A/T)GANA(A/T)GAA-3' and AD3: 5'-(A/T)GTGNAG(A/T)ANCANAGA-3' in combination with three nested right border-specific primers (RB-123: 5'-GCATGCAAGCTTGGCACTGG-3', RB-124: 5'-TGAGACCTCAATTGCGAGC-3' and RB-86: 5'-TCGGGCCTAACTTTTGGTG-3') or three nested left border-specific primers (LB-156: 5'-CCTATAAATACGACGGATCG-3', LB-155: 5'-ATAACGCTGCGGACATCTAC-3' and LB-154: 5'-TGATCCATGTAGATTTCCCG-3') (Campisi *et al.,* 1999). The primary PCR reaction was carried out in 20 µl with 1 µg TJ995 gDNA, 1 unit of *Taq* DNA polymerase, 2 µl 10x PCR buffer, 1.6 µl 25 mM $MgCl_2$, 2 µl 10 mM dNTPs, 4 pmol of LB-156 or RB-123 primer and 60 pmol of AD primer. Cycling parameters for the first round were as follows: (1) 95°C for 2 min; (2) 5 cycles of 94°C for 30 s, 62°C for 1 min, and 72°C for 2.5 min; (3) 1 cycle of 94°C for 30 s, 25°C for 3 min, ramp to 72°C in 3 min and 72°C for 2.5 min; (4) 15 cycles of 94°C for 10 s, 68°C for 1 min, 72°C for 2.5 min, 94°C for 10 s, 68°C for 1 min, 72°C for 2.5 min, 94°C for 10 s, 44°C for 1 min, and 72°C for 2.5 min; and (5) 72°C for 5 min. The primary PCR product was diluted 1:50 in water and 1 µl of the diluted primary PCR product was used for the secondary PCR reaction, in 20 µl with 1 unit of *Taq* DNA polymerase, 2 µl 10x PCR buffer, 1.6 µl 25 mM $MgCl_2$, 2 µl 10 mM dNTPs, 4 pmol of LB-155 or RB-124 primer and 40 pmol of AD primer. Cycling parameters for the second round were as follows: (1) 95°C for 2 min; (2) 15 cycles of 94°C for 10 s, 64°C for 1 min, 72°C for 2.5 min, 94°C for 10 s, 64°C for 1 min, 72°C for 2.5 min, 94°C for 10 s, 44°C for 1 min, and 72°C for 2.5 min; and (3) 72°C for 5 min. The secondary PCR product was diluted 1:50 in water and 1 µl was used for the tertiary PCR reaction, in 20 µl with 1 unit of *Taq* DNA polymerase, 2 µl 10x PCR buffer, 1.6 µl 25 mM $MgCl_2$, 2 µl 10 mM dNTPs, 4 pmol of LB-154 or RB-86 primer and 40 pmol of AD primer. Cycling parameters for the third round were as follows: (1) 95°C for 2 min; (2) 25-30 cycles of 94°C for 15 s, 44°C for 1 min, 72°C for 2.5 min, and (3) 72°C for 5 min. 20 µl of the secondary and third PCR product were loaded on a 3% agarose gel, and the fragments of the third PCR reactions that were slightly reduced in size compared to the secondary PCR fragments were isolated and directly sequenced.

Inverse PCR was performed as described by Long *et al.* (1993). Genomic DNA was digested with XbaI and ligated under dilute conditions in order to generate circular fragments. PCR with the RB and LB primers was then used to amplify DNA flanking the insertion site. 2 µl of the DNA was used in a PCR reaction of 50 µl with 1 unit of *Taq* DNA polymerase, 5µl 10x PCR buffer, 4 µl 25 mM $MgCl_2$, 5 µl 10 mM dNTPs, 10 pmol of the RB-86 and RB-96 (5'- AGTGCCAAGCTTGCATGC -3') primers or LB-156 and LB-31 (5'- TTGTAACGCGCTTTCCCACC -3') primers. Cycling parameters were as follows: (1) 95°C for 5 min; (2) 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 4 min; (3) 10 min 72°C. The PCR product was loaded on a 1% agarose gel and the fragments were isolated and cloned into pGEM-Teasy (Promega) according to the manufacturer's protocol and subsequently sequenced.

The T-DNA insertion sites within gene At5g04480 in line TJ995 and SALK_033889 were confirmed by direct PCR amplification using the T-DNA border-specific primers (RB-86 and LB-154 for TJ995 and pROK2 RB 5'- AGCAGGGACTCGCGGTGATT -3' and pROK2 LB 5'-GCGTGGACCGCTTGCTGCAACT-3' for the SALK line) in combination with the At5g04480 gene-specific primers flanking the found TJ995 T-DNA insertion (5'- CTCGTAAACGCCTTTCCAGTGT-3' and 5'- CTTCTGGACAAGATTAGACATCGATG-3') or flanking the annotated SALK T-DNA insertion (5'-TCTGATGGCAGCAAAATTTGATACC -3' and 5'- TTGGCATCCCTATCATAACACCTGA -3'), respectively.

Nucleotide sequence analysis

Sequence reactions were prepared with the Beckmann Quick Start kit according to the manufacturer's protocol. Reaction products were sequenced on a Beckman CEQ 2000 automated sequencer and interpreted using Beckmann software. Nucleotide sequences were analyzed using Blastn (Altschul *et al.,* 1990) and the vector NTI 6.0 program (Infomax).

Southern blot analysis

10 µg gDNA was digested overnight with the restriction enzyme KpnI, XbaI or BglII and electrophoretically separated overnight on 0.8% agarose gels. The DNA gel was treated as described by Sambrook *et al.* (1989), blotted onto a Hybond-N nylon membrane by capillary transfer in 10x SSC following the instruction guide of Amersham (1993) and fixed by baking at 80°C for two hours. The blots were pre-incubated in hybridization buffer (6x SSC, 0.5% (w/v) SDS, 5x Denhardts solution and 0.1 mg/ml denatured herring sperm DNA) for 4h at 65°C. Each blot was hybridized with a T-DNA-specific probe (GUS or KAN fragment) or with the At5g04480-specific probe. The GUS and KAN T-DNA probes were PCR products from the pD991 plasmid using the primers GUS forward primer 5'-GGGCAGGCCAGCGTATCGTG -3' and GUS reverse primer 5'- GTCCCGCTAGTGCCTTGTCCAGTT -3' and the KAN-forward primer 5'- CAGACAATCGGCTGCTCTGATGC -3' and KAN-reverse primer 5'-CGTCAAGAAGGCGATAGAAGGCG -3', respectively. The At5g04480-specific probe was a PCR product from Col-0 gDNA, using the gene-specific primers 5'- CTCGTAAACGCCTTTCCAGTGT-3' and 5'- CTTCTGGACAAGATTAGACATCGATG-3'. 25 ng DNA probe was ^{32}P-labeled by random primed labelling (Feinberg and Vogelstein, 1983) and hybridized to the DNA blot, overnight at 65°C. Also at 65°C, the filter was washed with an increasing stringency up to 0.2xSSC/0.1% (w/v) SDS, and Kodak X-omat AR Scientific Imaging Films were exposed to the blot with intensifying screens at -80°C, or a phosphoimager screen was exposed (Biorad). All autoradiograms were digitized and the contrast and brightness were adjusted with Adobe Photoshop. When the blot was used for hybridization with a second probe, the blots were stripped by incubation in boiling 0.1% SDS cooling to room temperature twice, and hybridized to the second radio-active probe as described.

Construction of the complementation construct and Arabidopsis transformation

In order to isolate the At5g04480 gene including the upstream 5'UTR and promoter regions and downstream 3' UTR region, BAC clone T32M21 DNA was digested with AgeI, and ligated into the XmaI restriction site of pPZP221 (binary vector containing the Gentamycin-resistance gene as plant selectable marker).The ligation product was transformed into *E. coli* DH5α cells, and clones containing the At5g04480 AgeI fragment were identified by bacterial colony blot hybridization with the At5g04480 gene-specific probe (as described by Sambrook *et al.*, 1989). To make sure that the At5g04480 promoter was not in the same orientation as the 35S promoter driving the plant selectable marker, the orientation of the insert was tested by restriction analysis and nucleotide sequence analysis, resulting in the complementation construct '+04480'. This binary vector construct was transferred to the *Agrobacterium tumefaciens* strain EHA101 (kindly provided by Dr. Stanton Gelvin (Purdue University) and Dr. Elisabeth Hood (Prodigene)), using freeze transformation (Chen *et al.*, 1994).

Arabidopsis Col-0 plants were transformed by the floral dip method, as described by Clough and Bent (1998). Briefly, nine plants were sown in 9x9 cm pots, incubated at 4°C for three days and transferred to a growth chamber. One week prior to transformation, the primary bolts were removed and the plants were watered well. One day prior to transformation the open flower buds of the secondary bolts were removed, and the plants were placed in a tray covered with a lid to maintain a humid environment. A 2 ml culture of *A. tumefaciens* with the binary construct were grown overnight at 28°C, 300 rpm. 1 ml of this culture was used to inoculate 250 ml LB with antibiotics in a 1 l flask, and grown at 28C at 300 rpm until OD600 is approximately 1.5. The cells are harvested at room temperature by spinning at 5000 rpm, and resuspended in infiltration medium, containing 0.5x MS salts (2.3 g/l) and 5% sucrose to a final OD600 of approximately 1. Immediately prior to incubation of the plants, 0.02% Silwet L-77 was added and the inoculum was transferred to a box. The inflorescences of the plants were submerged for 5 seconds in the inoculum, and the plants were placed horizontally in a closed plastic bag overnight. The next day the bags were opened and the plants were placed vertically within the bag. After one week, the plants were watered as normally until the T1 seeds were ready to be harvested from the dried siliques.

T1 seeds were grown on agar plates containing 0.5x MS salt including B5 vitamins (pH 6.0), MES buffer, 0.8% (w/v) select agar and 110 mg/l Gentamycin. Resistant seedlings were transplanted to soil

and segregation analysis of the Gentamycin resistance was performed to isolate plants containing a single T-DNA insertion (Col+04480). TJ995 and SALK plants were pollinated by Col+04480 and the segregation of the T-DNA insertion in At5g04480 (Kanamycin resistance) as well as the segregation of the complementing gene (linked to the Gentamycin resistance) was tested in two generations after self pollination and after reciprocal crosses with Col-0 plants.

Results

Screening a T-DNA population for gametophytic mutants

An enhancer trap T-DNA population of Columbia plants with the *glabra1* mutation, Col-6 *(gl1)* (Campisi *et al.,* 1999) was screened for gametophytic mutants, which can be detected by a distorted Mendelian-segregation of the NPTII selectable marker. 379 independent mutant lines were tested for segregation distortion. Initially, five lines were isolated that had a Kanamycin resistance to sensitive (R:S) ratio in agreement with a 1:1 ratio (X^2 goodness-of-fit analysis, $P>0.05$; 86-148 seedlings per line). However, after more elaborate selfing and reciprocal crossing tests only one of these lines, line TJ995, remained to consistently show the 1:1 segregation distortion, which was caused by a defect in the male T-DNA transmission alone, as the female T-DNA transmission appeared to be normal. These preliminary results suggested that line TJ995 was a male gametophytic mutant, and the genetics and genomic T-DNA organization of this line was further examined to identify the affected male gametophytic gene.

Segregation distortion in TJ995

The T-DNA segregation distortion of line TJ995 was analyzed in several generations and after two back crosses (BC) to wild-type plants. Table 1 shows that self-fertilization of the generations T3, T4, and the F2 generations of BC1 and BC2 resulted in a segregation of 1:1 Kanamycin R:S offspring. Female T-DNA transmission efficiency (TE) was unaffected, with an efficiency of 100% after TJ995 pollination with wild-type pollen. Male transmission was analyzed by pollination of emasculated Col-6 *(gl1)* plants or by pollinating *male sterility 1 (ms1)* Lansberg erecta plants with TJ995 pollen of the T4 generation and the F3 generation of BC1 (table 1). The T-DNA transmission through pollen was severely reduced, though crosses of wild-type flowers with TJ995 still resulted in 11 (out of 870) and 14 (out of 863) resistant seedlings, which corresponded to 1% male TE. These resistant plants were respectively called male BC1 (MBC1) and male BC2 (MBC2), since they originated from backcrosses using TJ995 as pollen donor. Table 1 also shows that the R:S ratio of the F3 generation of BC1 plants was 1.19. Although this ratio was not consistent with the expected 1:1 ratio, it came near to the ratio one would expect after self pollination, with 100% female TE plus 1% male TE. Thus, the ability to transmit the T-DNA through pollen, though strongly diminished, is likely to be the reason for this result. In fact, the R:S ratio after self pollination of the other generations tested (T3, T4 and the F2 generations of BC1 and BC2) were also consistent with this 1:1 + 1% R:S ratio.

The fact that still 1% of the pollen carrying a T-DNA can transmit this T-DNA to the offspring suggests that the T-DNA insertional mutation of the gametophytic gene does not give a fully penetrant phenotype. Alternatively, if multiple T-DNA

insertions were present some of the T-DNA copies could have been separated from the male gametophytic mutation by recombination. The unlinked T-DNA then would be inherited by the offspring independently of the gametophytic mutation. If this were true, plants with an unlinked T-DNA would have a normal Mendelian-segregation of the T-DNA, giving rise to 3:1 R:S seedlings. To test these hypotheses, the R:S segregation of seven of the MBC1 resistant plants were analyzed (table 1). Self fertilization resulted in 1:1 R:S ratio, and the male TE after pollination of Col-6 *(gl1)* or *ms1* plants was 1%. Thus, these plants still showed a male gametophytic defect, which indicated that the male gametophytic mutation caused by the T-DNA insertion is not fully penetrant, and that some mutant pollen tubes still were able to reach the ovules and support fertilization, producing viable, Kanamycin-resistant embryos.

Table 1 : Kanamycin resistance segregation in TJ995

generation	T3	T4	BC1		BC2	ratio [a]	TE [b]
			F2	F3	F2		
Selfing	0.90 (409 n=1)	1. 09 (7983 n=83)	0.97 (8816 n=83)	–	1. 10 (2349 n=8)	**1:1 or 1:1+1%**	
	–	–	–	1.19 (2030 n=13)	–	**1:1+1%** [c]	
TJ995 x Col-6*(gl1)*	n.a.	1.08 (836 n=34)	n.a.	1.01 (573 n=8)	n.a.	**1:1**	**100%**
Col-6*(gl1)* or *ms1* x TJ995	n.a.	0.01 (870 n=33)	n.a.	0.02 (863 n=8)	n.a.		**1%**
MBC1[d] selfing	–	–	0.97 (2063 n=7)	–	–	**1:1**	
Col-6*(gl1)* or *ms1* x MBC1	–	–	0.02 (817 n=7)	–	–		**1%**

Kan^R:Kan^S segregation after selfing and reciprocal crosses with Col-6*(gl1)* or *ms1* plants. In brackets are the total of seeds per generation (X^2 homogenity test, P>0.05), n is the total number of sister plants tested. a) X^2 goodness-of-fit P>0.05 unless stated otherwise b) T-DNA transmission efficiency through male or female gametes, TE=R/Sx100%, c) X^2 goodness-of-fit P>0.01. d) male backcross (MBC1): resistant progeny of Col-6*(gl1)* plants pollinated by TJ995, n.a. not analyzed.

Identification of the T-DNA insertion site

Inverse PCR and TAIL-PCR analyses to identify the T-DNA insertion site in TJ995 resulted in a complex pattern of PCR fragments, probably due to the presence of multiple T-DNA copies. One Inverse PCR fragment containing the left border (LB) flanking sequence was isolated and its sequence analysis showed homology to the gene At5g04480 on chromosome V (The *Arabidopsis* Genome Initiative, 2000). PCR analysis with gene-specific and T-DNA border-specific primers revealed that the insertion contained at least two T-DNA copies, in head-to-head orientation, and these two LB flanking sequences were located at position 29020 and 29033 of the BAC clone T32M21. The T-DNA insertion is located in the

predicted last intron (intron 13) of gene At5g04480, and a 13 base pairs deletion is present at the insertion site when compared to the sequence available at GenBank (*http://www.ncbi.nlm.nih.gov/*).

Southern blot analysis was performed to investigate the number of T-DNA copies present in the genome of TJ995. To this end, genomic DNA (gDNA) was digested with KpnI or XbaI, which both have a unique restriction site within the T-DNA, and hybridized with a T-DNA specific KAN- or GUS-probe (figure 1a). Figure 1c shows six KpnI fragments hybridized to the KAN probe and five fragments with the GUS probe, which suggested the presence of at least five T-DNA copies. A single T-DNA copy is 5.4 kb long and therefore fragments of this size that hybridized to both the KAN and GUS probe could correspond to a tandem repeat of two T-DNA copies (indicated by the asterisk in figure 1c). Southern blot analysis using XbaI (which kept the GUS and KAN genes within the same restriction fragment) resulted in five fragments visible after hybridization with the KAN probe. Theoretically these five fragments should all hybridize with a GUS probe, however, one fragment does not do so, indicating that one T-DNA may be truncated, lacking the GUS gene (arrowhead, figure 1c). However, as this putative truncated T-DNA fragment would still be carrying the NPTII gene, it would presumably still be contributing to the Kanamycin resistance phenotype. The high intensity of the smallest XbaI fragments may be due to two T-DNA copies in fragments of approximately the same size, which could explain the lower number of hybridizing fragments on the XbaI blot compared to the KpnI blot.

To analyse whether all five or six T-DNA copies were located within the At5g04480 gene, gDNA of TJ995 and control plants were digested with BglII, a restriction enzyme with no restriction sites within the T-DNA region, and hybridized with the KAN probe or gene-specific probe (figure 1b and d). The gene-specific probe was chosen to cover the entire gene, from promoter to 3' UTR, comprising almost the entire sequence between gene At5g04470 and At5g04490, the annotated genes upstream and downstream of At5g04480 (figure 1b). Two separate blots were hybridized, stripped and re-hybridized reciprocally with the KAN probe and the gene-specific probe, resulting in similar hybridization patterns. Figure 1d shows that the gene-specific probe hybridized with three fragments of the expected sizes 1414, 4911 and 6489 bp of wild-type DNA (figure 1b). Also with TJ995 these three wild-type gene fragments were visible, as expected since TJ995 is a hemizygous mutant. Additionally, two larger fragments of approximately 10 and 14 kb hybridized with the gene-specific probe in TJ995. With the KAN probe, three fragments were revealed in the mutant, suggesting that the five or six single T-DNA copies shown in figure 1c were organized in three T-DNA insertion sites. Two of these KAN probe fragments corresponded in size with the two 10 kb and 14 kb fragments hybridizing with the gene-specific probe (arrows in figure 1d), indicating that the change in size compared to the wild-type fragments is due to the T-DNA

insertion within this gene. However, the third KAN fragment (of ~12 kb) is not revealed using the gene-specific probe. The genetic data suggests that this T-DNA insertion is probably closely linked to gene At5g04480 on chromosome V, since Kanamycin resistance analysis showed no recombination events between the separate T-DNA copies.

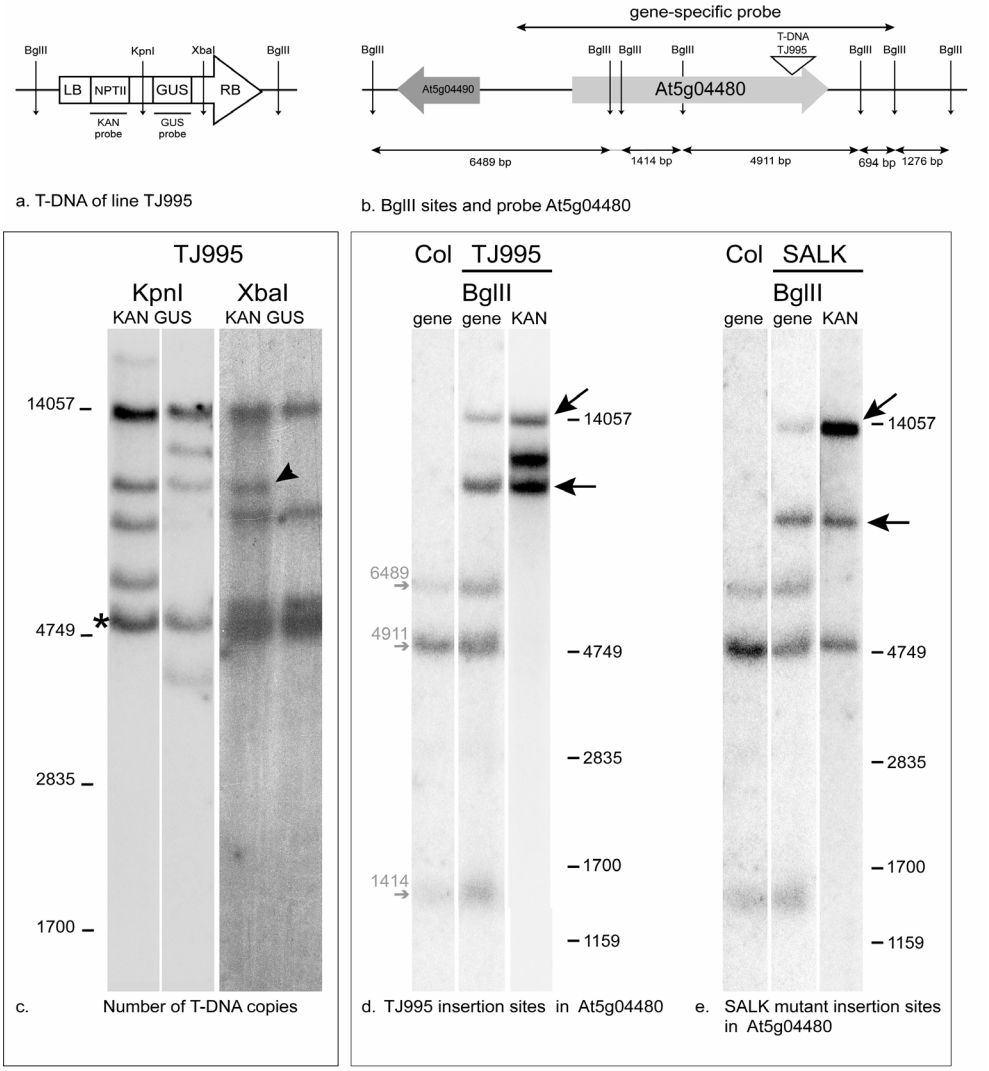


Figure 1: Southern blot analysis of the T-DNA insertions

10 µg of genomic DNA of TJ995, wild type (Col) and the SALK mutant was digested with KpnI, XbaI or BglII, and hybridized with a T-DNA specific probe (KAN or GUS) or with a gene-specific probe. a. the KpnI, XbaI and BglII restriction sites and the two T-DNA probes within the T-DNA of TJ995, b. the BglII restriction sites and the gene-specific-probe of At5g04480, c. Southern blot of TJ995, hybridized with the GUS or KAN-probe. * 5.4 kb fragment could indicate a T-DNA repeat. Arrowhead: putative truncated T-DNA, d. Col and TJ995 Southern blot. e. Col and SALK Southern blot. Arrows in d. and e.: T-DNA insertion within gene At5g04480.

In order to localize all three T-DNA insertion sites and to exclude the possibility of other mutations, additional TAIL-PCR experiments were performed and the PCR products were sequenced using T-DNA border-specific primers. A summary of the sequence homology-analysis of the flanking border sequences is shown in table 2. Some of the results confirmed earlier data obtained by PCR and southern blot analysis, which were two left borders adjacent to the At5g04480 flanking sequences and a tandem repeat from the right border into the left border. Most of the other flanking sequences showed high homology with vector sequences and presumably are caused by read-through of the borders during T-strand formation in *Agrobacterium*. However, not all vector sequences seemed to be derived from the used binary vector pD991, but were more likely caused by mistakes during the T-DNA integration process (table 2, LB4, RB4 and RB5). Intriguingly, one RB flanking sequence (RB5) showed strong homology to At1g11280, an S-locus gene on chromosome I, though this flanking sequence could be the result of an aborted T-DNA integration with a small piece of RB sequence remaining at the insertion site. Taken together, we can conclude that multiple T-DNA copies are present in line TJ995, and are integrated into the plant genome in a complex manner. No other mutated genes besides At5g04480, however, could be identified using these methods.

Table 2: Flanking border sequence analysis

Flank. Border	Sequence homology		Technique
LB1	At5g04480, BAC T32M21, position 29033-29593	566 bp, 95%	inverse PCR, TAIL-PCR (AD1) and direct PCR
LB2	At5g04480, BAC T32M21, position 28696-29020	270 bp 96%	direct PCR
LB3	beyond the LB sequence plasmid pD991	225 bp, 100%	TAIL-PCR (AD2)
LB4	homology to VirE2 gene	623 bp, 97%	TAIL-PCR (AD2)
RB1	tandem repeat LB sequence T-DNA	525 bp, 99%	TAIL-PCR (AD3)
RB2	LB vector sequence (no homology to beyond LB sequence pD991)	289 bp, 100%	TAIL-PCR (AD2)
RB3	replication of origin and BOM site (no homology to beyond LB sequence pD991)	770 bp, 95%	TAIL-PCR (AD1)
RB4	pieces of RB sequence (filler DNA?)	35 bp, 100%	TAIL-PCR (AD2)
RB5	pieces of RB sequence (filler DNA?) as in RB4, and homology to At1g11280 (S-locus gene)	35 bp, 100% 110 bp 100%	TAIL-PCR (AD2)

Summary of the flanking border sequences found by Inverse PCR, TAIL-PCR and direct PCR. TAIL-PCR is performed using three different random primers (AD1, AD2 or AD3). The sequence homology was analysed using NCBI nucleotide blast (non-redundant database) and TAIR nucleotide blast (AGI genes database). Sequences with homology to a vector sequence were aligned to part of the binary vector pD991 to test the sequence identity. The total length of the sequence that shows homology and the percentage of homology are indicated.

Independent alleles and complementation of the At5g04480 gene

To test whether the segregation distortion in TJ995 is due to the mutation of gene At5g04480, an independent T-DNA insertion line of At5g04480 was tested for the segregation distortion phenotype. Furthermore, we tested whether the introduction of a wild-type copy of At5g04480 was able to complement the male gametophytic mutation and restore the normal Mendelian-segregation ratio in the T-DNA mutants.

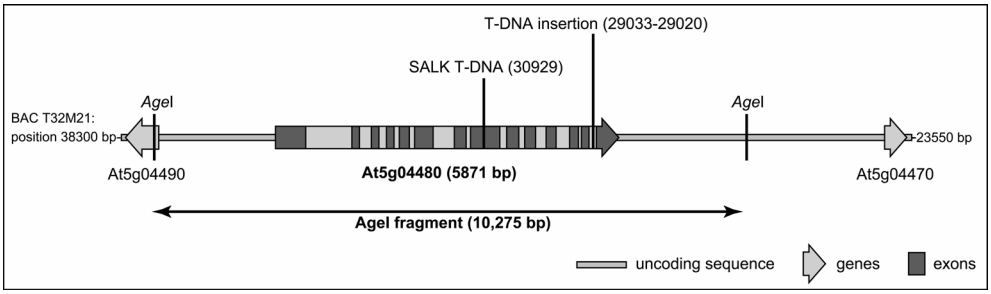


Figure 2: T-DNA insertion sites in gene At5g04480
The identified T-DNA insertion of TJ995 is inserted in intron 13 and the T-DNA of SALK line 033889 is inserted in exon 8 (the exact position on the BAC clone is indicated between brackets). The 10.2 kb AgeI restriction fragment was used for complementation analysis.

Segregation distortion of the independent SALK T-DNA mutant

SALK line 033889 in the SIGNAL SALK database was reported to have a T-DNA insertion in gene At5g04480, and this insertion in exon 8 was confirmed by PCR. A representation of the wild-type gene and the T-DNA insertion sites of the two independent mutants is shown in figure 2. The T-DNA of the SALK mutant also contains the NPTII gene as selectable marker. Segregation analysis of the Kanamycin resistance in progeny after self pollination showed a R:S ratio of 1:1 (table 3). Reciprocal crosses showed that, like in line TJ995, this segregation distortion is caused by a strongly reduced male T-DNA transmission efficiency of 1%, while female transmission is unaffected (table 3). Segregation analysis of MBC1 plants revealed a 1:1 R:S segregation, indicating that the male gametophytic mutation is not fully penetrant in this mutant line, as it was already shown for TJ995. Southern blot analysis was performed as described for TJ995, using restriction enzyme BglII, which does not have a restriction site in the pROK2 T-DNA of the SALK line (figure 1e). With the gene-specific probe, two additional fragments, as compared to wild type, are present in the SALK mutant corresponding to the insertion of the T-DNA in gene At5g04480 (indicated by the arrows). Also in this mutant, one T-DNA fragment was shown to have originated from a T-DNA insertion located elsewhere.

Table 3: Kanamycin resistance segregation in the SALK mutant

generation	TJ995	SALK	ratio [a]	TE [b]
Selfing	1.19 (2030 n=13)	1.14 (4062 n=27)	**1:1+1%** [c]	
Mutant x Col	1.01 (573 n=8)	1.18 (368 n=7)	**1:1**	**100%**
Col or *ms1* x Mutant	0.02 (863 n=8)	0.02 (1383 n=24)		**1%**
MBC1[d] selfing	0.97 (2063 n=7)	1.10 (931 n=19)	**1:1**	

Kan^R:Kan^S segregation after selfing and reciprocal crosses with Col-0, Col-6*(gl1)* or *ms1* plants. In brackets are the total of seeds per generation (X^2 homogenity test, P>0.05), n is the total number of sister plants tested. a) X^2 goodness-of-fit P>0.05 unless stated otherwise, b) T-DNA transmission efficiency through male or female gametes, TE=R/Sx100%, c) X^2 P>0.01. d) male backcross (MBC1): resistant progeny of Col-O or Col-6*(gl1)* plants pollinated by the mutant plants.

At5g04480 complementation test

To test whether the correct gene was isolated, molecular complementation was performed. A wild-type copy of At5g04480 was introduced in both line TJ995 and the SALK 033889 mutant and these transformed mutants were analyzed for restored Mendelian segregation. The construct used for complementation (named +04480) consisted of the ~10 kb AgeI fragment of BAC AtT32M21, which comprised the At5g04480 coding sequence with 2151 bp upstream the ATG as promoter region, and 2220 bp sequence downstream the stopcodon (figure 2). This fragment was ligated into the T-DNA of binary vector pPZP221, which confers Gentamycin resistance in plants. Col-O plants were transformed with the complementation construct and the transformants were screened for the number of T-DNA insertion loci by Gentamycin segregation analysis. One transformant (called Col+04480) contained a single T-DNA locus, and this line was crossed with TJ995 and the SALK mutant.

Figure 3 shows a schematic representation of the crossings performed and the expected Kanamycin and Gentamycin resistance segregation in the complementation test. Pollination of TJ995 or the SALK mutant with 'Col+04480' resulted in F1 seedlings carrying both T-DNAs (genotype Kan^R/ +; Gen^R/ -) and thus were resistant to both Kanamycin and Gentamycin selection on seed germination medium. The plants were self fertilized twice and the Kanamycin resistance segregation of the offspring was analyzed. After the first selfing the F2 plants were hemizygous for the complementation T-DNA (Gen^R/ -), and the overall Kanamycin R:S ratio was changed from 1:1 to 2:1 (figure 3 and table 4). This indicated that pollen carrying both the mutating Kan^R T-DNA and the complementation Gen^R T-DNA could transmit Kanamycin resistance to the progeny

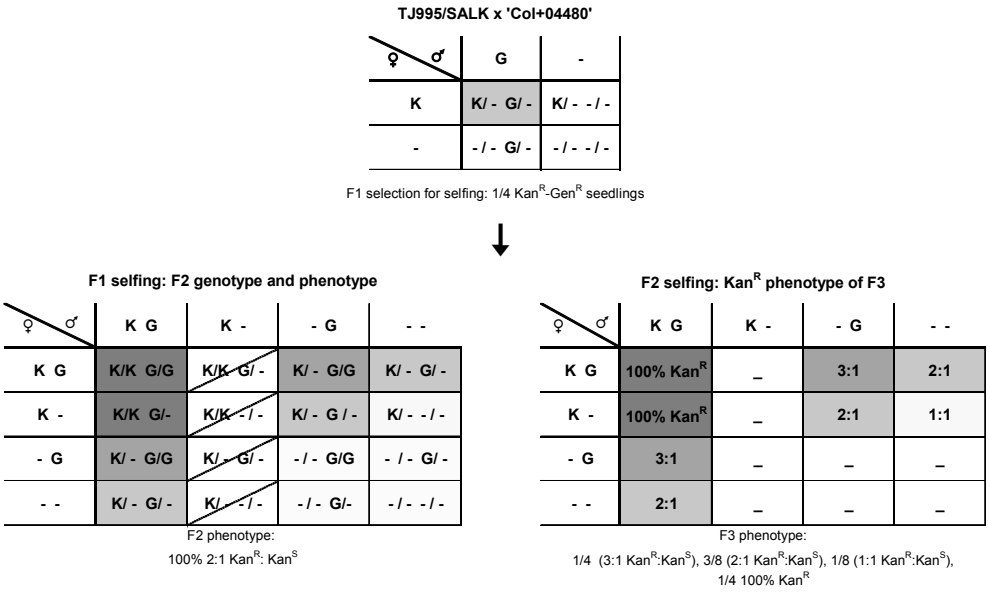


Figure 3: Complementation crossing scheme and expected Kanamycin resistance phenotypes
Expected genotypes and Kanamycin resistance phenotypes of the F2 and F3 progeny after successful complementation of the male gametophytic mutation. The mutant lines (Kan^R) were crossed with 'Col+04480', a transformant with a single insertion of the complementation T-DNA (Gen^R). The Kan^R and Gen^R T-DNAs are assumed to be unlinked. K: Kan^R, G: Gen^R, - : no T-DNA

and that Kan^R T-DNA transmission through pollen without the complementation construct was still strongly reduced. This was also confirmed by the increase in male T-DNA transmission from 1% to 50% when *ms1* plants were pollinated by TJ995 plants that were heterozygous for the complementation construct (Gen^R/ -) (table 4). The F3 generation after the second self fertilization contained plants which were hemizygous (Gen^R/ -) or homozygous (Gen^R/Gen^R) for the complementation construct. The Kanamycin resistance segregation in the homozygous Gen^R plants was fully restored to 3:1 R:S (figure 3 and table 4). Some F2 plants gave rise to 100% Kanamycin resistant seedlings (data not shown). These plants resulted from fertilization of an egg cell with a Kan^R;Gen^R or Kan^R;Gen^S genotype by pollen with the Kan^R;Gen^R genotype (figure 3), and this clearly indicated that the male Kan^R T-DNA transmission was restored.

The complementation test has shown that the distorted T-DNA segregation of both TJ995 and the SALK mutant could be restored to the Mendelian 3:1 ratio with the introduction of functional At5g04480 gene. Therefore, we can conclude that the male gametophytic defect in the TJ995 and in the 033889 SALK mutants is caused by the T-DNA insertional mutation of gene At5g04480. Thus, although additional T-DNA insertions are present in both mutant lines, these do not affect the function of the male gametophyte.

Table 4: Complementation of TJ995 and the SALK mutant with a functional At5g04480 gene

	genotype +4480	F2 Kan R:S	ratio [a]	*ms1* x F1 ♂	male TE [b]	F3 Kan R:S	ratio [a]
TJ995 x Col+04480	- / -	1.02 (4468 n=14)	**1:1**	0.00 (497 n=3)	**1%**	0.77 (209 n=3)	**1:1**
	Gen^R/ -	1.97 (4236 n=16)	**2:1**	0.40 (507 n=4)	**50%**	2.25 (1716 n=20)	**2:1**
	Gen^R/Gen^R	—	—	—	—	2.89 (802 n=9)	**3:1**
SALK x Col+04480	- / -	1.26 (1333 n=23)	**1:1+1%** [c]	n.a.	—	1.01 (706 n=8)	**1:1**
	Gen^R/ -	2.00 (1430 [d] n=22)	**2:1**	n.a.	—	2.00 (902 n=10)	**2:1**
	Gen^R/Gen^R	—	—	—	—	3.01 (1294 n=15)	**3:1**

Kan^R:Kan^S segregation of F1 and F2 progeny of TJ995 or SALK plants pollinated with a transformant (Col+4480) containing a single T-DNA with a wt copy of At5g04480. The analyzed plants were heterozygous or homozygous for the complementation T-DNA (Gen^R), or did not have this T-DNA (- / -). In brackets are the total number of seedlings tested per generation (X^2 homogenity test, P>0.05), n is the total number of independent crosses tested. a) ratio X^2 goodness-of-fit P>0.05 unless stated otherwise, b) Transmission efficiency through pollen, TE=R/S x100%, c) ratio=1:1+1%(TEmale), X^2 goodness-of-fit P>0.01, d) X^2 homogenity test, P=0.016, n.a. not analyzed.

At5g04480 codes for a GT1 glycosyl transferase

No function has been annotated so far to the 1050 amino acids long gene product of At5g04480 (NP_568137) and DNA sequence homology analysis did not show a significant homology to genes present in GenBank. Protein sequence analysis, however, showed homology with one other *Arabidopsis* protein (NP_192030, product of gene At4g01210, 34% identity) and with two proteins of *Oryza sativa* (figure 4). The rice protein NP_922601 showed the highest homology, with 43% identity, but also the function of this protein is not known. At amino acid level, the homology was randomly distributed, but it was strongest at the C-terminal end of the protein. Interestingly, the intron-exon boundary structure of the genes coding for the four homologous proteins appeared to be highly conserved (arrows in figure 4). The protein of At4g01210 has been annotated to be a glycosyl transferase family I member, as a result of the high homology with a conserved domain of glycosyl transferase group 1 proteins (glycos_transf_1, pfam00534, Bateman *et al.,* 2004). Figure 5 is a representation of the predicted At5g04480 protein that also showed homology with this glycos_transf_1 domain, contained a predicted N-terminal transmembrane domain (Aramemnon database, Schwacke *et al.,* 2003) and has a domain homologous to the ATP/GTPase binding site A (P-loop) (Prosite, *http://www.expansy.org*). Both the glycosyl transferase domain and

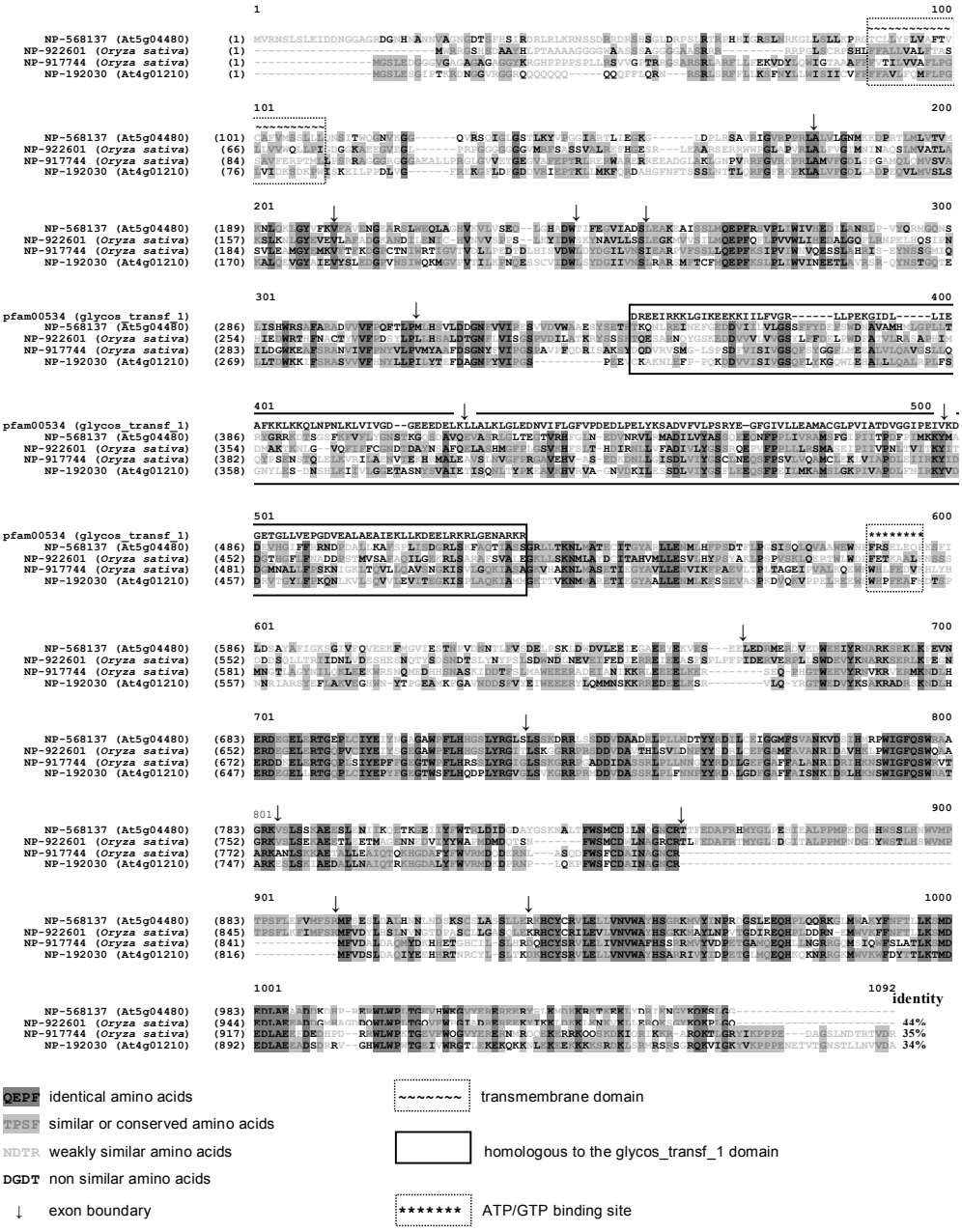


Figure 4: Protein homologues of the At5g04480 gene product

Multiple sequence alignment of the At5g04480 protein, the *Arabidopsis* homologue (At4g01210) and the two rice orthologues. Identities shown are the homologies with NP-568137 (At5g04480). NP-917744 (Oryza sativa) and NP-192030 (At4g01210) have a similarity of 45%.

the N-terminal transmembrane domain are predicted to be present in all three homologous proteins. This predicted protein structure resembles a type II membrane protein, with an N-terminal cytoplasmic tail. Although the At5g04480 gene product is not included in the Carbohydrate-Active enZYmes (CAZy) database (Coutinho and Henrissat, 1999) that contains most glycosyl transferases, the secondary structure of At5g04480 strongly suggested a functional role as a glycosyl transferase. Recently, a bioinformatics-based survey has identified the At5g04480 gene product as one of the glycosyl transferase family 1 members that were lacking in this database, thus confirming our hypothesis about the putative function of this protein (Egelund *et al.,* 2004).

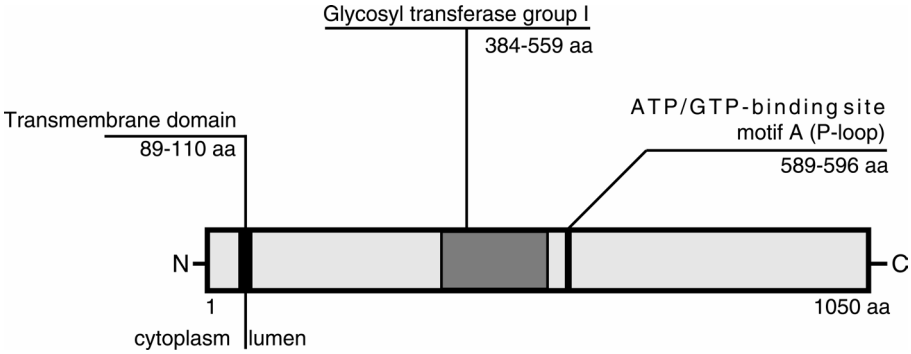


Figure 5: Protein domain analysis

The relatively high homology between the gene products of At5g04480 and At4g01210 might suggest that the two proteins have a similar function in *Arabidopsis*. To investigate whether At4g01210, like At5g04480, is important for proper functioning of the male gametophyte, two SALK T-DNA insertional mutants of this gene were ordered and analyzed. Unfortunately, PCR analysis of the T-DNA insertion sites of these mutants did not confirm the annotated insertions in gene At4g01210.

Discussion

A male gametophytic mutant, line TJ995, was selected in a segregation distortion assay. Male T-DNA transmission efficiency (TE) was reduced to 1%, while female TE was 100%. Genetic analysis of the resistant offspring originating by male T-DNA transmission after pollinating wild-type plants with TJ995 pollen showed that these plants had the same defect in male T-DNA transmission (TEmale=1%), indicating that the T-DNA induced gametophytic mutation severely affects pollen function, but is not fully penetrant.

Southern blot analysis has shown that the T-DNA integration pattern in line TJ995 is complex: five or six T-DNA copies are present within the genome, one of which is truncated while several T-DNA copies are orientated in a tandem and an inverted repeat. Tandem and inverted repeats are common features found in T-DNA insertion lines (Gheysen, 1991). Most likely, the truncated T-DNA still contained a functional NPTII gene (as identified on southern blot by hybridization with the KAN-probe) and therefore we can assume that all T-DNA copies contributed to the Kanamycin phenotype of line TJ995. The T-DNA copies were located at three different insertion sites, but no Kanamycin resistant plants without the segregation distortion phenotype has been observed, suggesting that all T-DNA insertions are linked to the male gametophytic mutation.

Inverse PCR and TAIL-PCR analysis showed that one of the T-DNA insertions is located in gene At5g04480, causing a 13 bp deletion. Such small deletions are often found following *Agrobacterium* T-DNA integration (Gheysen, 1991), and is presumably caused by the removal of plant DNA by the plant repair excision mechanism (Tinland, 1996). Analysis of the other flanking sequences did not result in additional insertion sites at chromosome V as was expected, but rather represented the various T-DNA integration errors described in literature. Most of these flanking sequences showed high homology with the pD991 vector sequence, which was presumably caused by read-through of the border sequences during T-strand formation within the *Agrobacterium* (Martineau *et al.,* 1994). One flanking left border sequence showed strong homology with VirE2, a virulence gene located on the helper plasmid of the *Agrobacterium*, and no explanation for this has been suggested so far. Right border flanking sequences showed the presence of short RB repeats, resembling filler DNA that results from slippage of the T-DNA during the illegitimate recombination process (Roth, 1989; Gheysen, 1991). Chromosomal rearrangements and duplications have been observed regularly after T-DNA integration (Castle *et al.,* 1993; Martineau *et al.*, 1994; Liu *et al.,* 1995; McKinney *et al.,* 1995; Wenck *et al.,* 1997; Nacry *et al.,* 1998; Tax and Vernon, 2001), and this might explain the RB flanking sequence with strong homology to an S-locus gene on chromosome I. However, since all T-DNA copies rendering Kanamycin resistance are linked to the gametophytic mutation on chromosome V, it is more

likely that this recovered right border fragment is not linked to the NPTII gene but result from an aborted T-DNA integration, leaving a fragment of the right border behind as footprint. The complex T-DNA integration pattern in TJ995 asks for thorough analysis of the mutant, and two different tests were performed to analyse whether the T-DNA insertional mutation in gene At5g04480, the only mutated gene identified so far, was causing the male gametophytic defect found.

An independent T-DNA insertion line of gene At5g04480, the SALK mutant 033889, also showed segregation distortion, a strong indication that the T-DNA insertion within At5g04480 is causing the male gametophytic defect. Unequivocal proof that the correct gene has been isolated however, was obtained by molecular complementation: we have shown that the T-DNA segregation of TJ995 and the SALK mutant was fully restored from 1:1 to 3:1 R:S when the plants were homozygous for a T-DNA carrying the wild-type At5g04480 allele. The other T-DNA insertions in both mutants thus, do not affect the male gametophytic function, and neither does the putative chromosomal translocation in TJ995. The defect in male T-DNA transmission is caused by the T-DNA insertional mutation in gene At5g04480 alone. It is intriguing though, that both mutants show 1% residual male transmission, as the T-DNA insertions in the two mutants are inserted at different places within the gene.

Although no function has been annotated to At5g04480, domain analysis of the predicted protein shows resemblance with a type II transmembrane protein, with a glycosyl transferase group 1 domain and an ATP/GTP A binding site, suggesting that the protein might be a glycosyl transferase. Group 1 glycosyl transferases transfer UDP, ADP, GDP or CMP linked sugars to various substrates, and this group is conserved across all kingdoms. Most glycosyl transferases are located in the Golgi apparatus, where they play a role in the production of glycoproteins or synthesis of polysaccharides (Keegstra *et al.,* 2001). These products have been shown to be important for pollen tube growth, as signal proteins or cell wall components, and a defect in either of these classes of molecules might cause the male gametophytic defect. The most homologous *Arabidopsis* protein is the gene product of At4g01210, which has been annotated as a glycosyl transferase family 1 member. The only two other homologous proteins present in GenBank are from *Oryza sativa*. Both protein homology and the fact that the intron-exon boundaries of the encoding genes were highly conserved through evolution, suggest that the *Arabidopsis* and *Oryza* proteins have a similar and important function. Unfortunately, no T-DNA insertional *Arabidopsis* mutant of gene At4g01210 was available to test its male T-DNA transmission efficiency. However, if the two genes would have been redundant in pollen we would not have found the strong defect in male T-DNA transmission in line TJ995.

To conclude, we have identified a male gametophytic mutant that has a mutation in gene At5g04480. The protein might function as a glycosyl transferase,

an enzyme that is generally located in the secretory pathway. Protein localization studies will show whether this is also the case for the gene product of At5g04480. Glycosyl transferases are very specialized enzymes that transfer sugar groups to specific substrate proteins to form glycoproteins or polysaccharides. Analysis of the mutant phenotype of the two mutant alleles could give important information on which process is affected in pollen-pistil interactions and fertilization, and will provide information about the putative function of this At5g04480 protein.

Chapter 4

Phenotypic analysis of the male gametophytic mutant *bursting pollen (bup)* shows a defect in pollen germination and pollen tube growth

Karin Hoedemaekers, Mieke Wolters-Arts, Yeni Coronado, Jan Derksen, David Twell and Titti Mariani

Abstract

T-DNA insertional mutants of *BURSTING POLLEN (BUP)*, a gene (At5g04480) encoding a putative glycosyl transferase protein, show a strong defect in male transmission of the T-DNA. This suggests that this protein plays an important role during pollen development or pollen tube growth. Glycosyl transferases of the GT1 family are transmembrane proteins with a single transmembrane domain, involved in the synthesis of carbohydrous cell wall components and glycoproteins. While pollen development and fertilization appeared normal in *bup* mutants, *in vitro* pollen tube growth analysis showed a defect in pollen germination, indicated by the presence of cytoplasmic extrusions lacking a plasma membrane and cell wall. The bursting of these pollen might be due to a defect in the structure of the intine layer or that of the germination plaque at the site of pollen tube emergence. *In vivo*, 2% of the mutant pollen succeeded in growing a pollen tube and achieving fertilization. These mutant pollen tubes, however, also have a defect in growth as is shown by competition analysis. Pectin synthesis, pectin modification and the presence of AGPs are all known to be associated with the intine layer during pollen germination, and a defective glycosylation step of any of these compounds could result in a decrease in intine rigidity which might explain the *bursting pollen* phenotype. The result that no homozygous mutant plants were identified, despite the strong effort to do so, suggests that *bup* mutants might be embryo lethal and is therefore also likely to be important for the development of cell walls of sporophytic tissues.

Introduction

During sexual reproduction in plants, the male gametophytes or mature pollen and pollen tubes transport the sperm cells to the ovules, where fertilization and seed development takes place. Pollen development in *Arabidopsis* follows the general pattern of pollen development seen in most angiosperms (Owen and Makaroff, 1995). The diploid microspore mother cell undergoes meiosis I and II, resulting in four haploid microspores positioned in tetrads. This tetrad stage is ended by degradation of the surrounding walls and release of the single microspores. The next stage in pollen development is the asymmetric mitotic division of the microspore which produces a large vegetative cell and a smaller generative cell. A second mitotic division of the generative cell is needed to form two sperm cells. In *Arabidopsis*, this division takes place during pollen development in the anther, resulting in tricellular mature pollen with two sperm cells positioned within the vegetative cell. In species with bicellular mature pollen this second division takes place within the growing pollen tube. Mature pollen is surrounded by a wall that consists of an inner layer (the intine) composed of pectin and cellulose, and an outer layer (the exine) that is made up of sporopollenin and is covered by a coat derived from the degenerated tapetum. The last step in pollen development is a partial dehydration of the pollen grains, largely mediated by evaporation upon anther dehiscence. In the self-fertile *Arabidopsis*, mature pollen are transferred to the stigma by direct contact. Upon pollen deposition, adhesion follows immediately and the pollen coat accumulates between the pollen grain and the stigma forming a 'foot' structure (Elleman *et al.*, 1992; Zinkl *et al.*, 1999). The pollen hydrates by water uptake from the stigma, followed by pollen germination. During pollen germination, the grain organizes its cytoplasm and cytoskeleton to support the extension of a single tube, and callose is deposited at the site of pollen tube emergence (Johnson and McCormick, 2001; Lalanne *et al.*, 2004). The pollen cytoplasm then breaches through the exine at the site of the apertures or through the interapertural exine walls, which is often the case in *Arabidopsis* (Edlund *et al.*, 2004). Pollen tubes show polarized cell growth, a specialized type of growth called tip growth. At the tip of the pollen tube, Golgi-derived vesicles fuse with the plasma membrane and secrete pectic material that will form the primary wall. The components of a secondary, inner pollen tube wall are synthesized by callose- and cellulose synthases at the plasma membrane flanking the tip (Ferguson *et al.*, 1998). The emerged pollen tube penetrates the stigma and grows through the pistil, until it emerges on the septum surface in the ovary, a process that is referred to as the progamic phase of pollen-pistil interactions. Within the ovary, multiple signals from the ovules guide the growing pollen tube to the embryo sacs until a pollen tube will penetrate the micropyle (Hülskamp *et al.*, 1995). Once the sperm

cells are released into the embryo sac, gamete fusion and embryo development takes place and fertilization has been achieved.

During the last decade, mutants of *Arabidopsis* each affected at any stage of pollen development or pollen tube growth have been described in literature (reviewed in Twell, 2002; McCormick, 2004). The male gametophytic mutants *bup-1/+* and *bup-2/+* of gene At5g04480, were identified by segregation distortion analysis of the T-DNA insertion (Chapter 3). In these mutants, the male T-DNA transmission is reduced to 1% of the wild-type efficiency. Domain structure analysis of the BUP protein suggested that it may be a glycosyl transferase of the GT1 family (protein number Q9LZ77, Egelund *et al.*, 2004). Glycosyl transferases are needed for the synthesis of glycoproteins and polyglycans, molecules involved in cell wall synthesis and also in signalling cascades. 25 different families exist in *Arabidopsis*, comprising 351 genes so far, indicating their specialized reaction with particular sugar residues and substrate molecules. So far, only a few glycosyl transferases have been suggested to be involved in the male gametophyte's function. Of these, only flavonol 3-O-galactosyl transferase, a pollen-specific glycosyl transferase that regulates the formation of flavonol glycosides, belongs to the GT1 family. Flavonols may be essential for pollen germination in *Petunia* and maize (Vogt *et al.*, 1995), but *Arabidopsis* mutants in the flavonol synthesis (*chsA)* did not have a defect in pollen germination, suggesting that flavonols are not essential for *Arabidopsis* pollen germination (Ylstra *et al.*, 1996). Other glycosyl transferases have been identified by T-DNA mutational analysis. Mutants of the cellulose synthase-like putative glycosyl transferase, AtCSLA7, show a reduction in the male transmission of the mutation (Goubet *et al.*, 2003). This gene was shown to be expressed ubiquitously and the homozygous mutant lines were embryo lethal, indicating that AtCSLA7 is crucial for overall cell wall synthesis in plant cells. Double mutants of the *SPEC* and *SPY* O-linked N-acetylglucosamine transferase genes, also show a reduction in the transmission of the mutations through the male gametophyte, in addition to embryo lethality (Hartweck *et al.*, 2002). Finally, the GT10 family member α-4-fucosyltransferase was suggested to be involved in pollen germination or pollen tube growth, though this was only based on its pollen-specific expression pattern (Joly *et al.*, 2002).

Considering the enormous amount of different cell wall material needed for tip growth, it is likely that many more glycosyl transferases are involved in pollen development and pollen tube formation. Here we investigate the function of the BUP glycosyl transferase in pollen development and pollen tube growth by analyzing the *bup* mutant phenotype. To this end, possible defects in pollen development, pollen germination and pollen tube growth were analyzed by light - and electron microscopy.

Material and methods

Plant material

Seeds of *Arabidopsis thaliana* L. ecotype Col-O and Col-6 (Col-0 with *glabra1* mutation, ABRC stock number CS8155) were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC). *Arabidopsis* plants were grown in a 3:1 mixture of soil and vermiculite and kept under greenhouse conditions with a 16 hours photoperiod, at 22°C and ambient relative humidity. The mutant line *bup-1/+* was isolated from a set of pooled Tom Jack enhancer trap T-DNA lines (line TJ995, ABRC stock numbers CS31086 and CS31087) in the Col-6 background. *bup-2/+* (SALK line 033889) was obtained from NASC. The *qrt-1* mutant and LAT52-GUS *Arabidopsis* transformants were kindly provided by Dr. David Twell. *bup-1/+* and *bup-2/+* seeds were surface sterilized by incubation for 2 minutes in 96% ethanol, followed by 5 minutes incubation in a solution of 40% bleach and 0.1% Tween-20. The seeds were washed at least 4 times with sterile dH_20, and pipetted onto selection plates in 0.1% select agar. Alternatively, the seeds were sterilized in a drop of 95% ethanol. Ethanol was allowed to evaporate overnight. Sterilized seeds were plated onto Kanamycin (50 mg/l)-supplemented medium (2.2 g/l MS salts, 0.5 g/l MES, pH 5.7 and 7 g/l select agar, pH 5.8). After 3 days at 4°C, plates were incubated under 12 h photoperiod at 21°C and one to two weeks later resistant seedlings were transferred to pots containing a 3:1 mixture of soil and vermiculite and grown under greenhouse conditions with supplemental lighting (16 hours of light at 22°C).

Transmission of the mutations through the male gametes was determined by manually pollinating wild-type (Col-0, Col-6*(gl1)* or male-sterile (*ms1*) Lansberg erecta plants with *bup-1/+* or *bup-2/+* pollen. Limited pollination experiments were performed by applying precisely 20 pollen grains onto the mature stigmas. Male transmission efficiency (TEmale) was calculated according to Howden *et al.* (1998). The expected number of gametes carrying the trait is taken to equal the number of gametes lacking it, assuming random segregation during meiosis and the absence of post- meiotic selection; therefore, TE (%)=(observed R/observed S) x 100.

In vitro pollen germination and pollen tube growth

For mature pollen analysis, mutant plants were crossed with Lansberg erecta *qrt1* (Preuss *et al*. 1994). Heterozygous *bup-1/+* or *bup-2/+* plants homozygous for the *qrt1* mutation produce tetrads that contain two wild-type and two mutant pollen grains, allowing direct morphological comparison of pollen phenotype. *In vitro* germination assays were performed as described in Derksen *et al.* (2002, Chapter 2). Mature pollen were collected by tapping open anthers on small pieces of Visking dialysis membrane (Serva, Germany). The membranes were boiled for 6 h in water and soaked in liquid medium before use. The membranes were placed on semi-solid medium (Hodgkin, 1983) consisting of 0.01% H_3BO_3 (Merck, Germany), 0.7% Bacto-Agar (Difco, Detroit, Mich.), 0.07% $CaCl_2 \cdot 2H_2O$ (Merck, Germany), 3.0% polyethylene glycol 4,000 (Duchefa,The Netherlands) and 20% sucrose (Mallinkrodt Baker, The Netherlands), pH 7. In some experiments, excised *Arabidopsis* pistils were added to the membranes. Pollen were germinated and grown in the dark at 27°C. Pollen tubes were photographed with a conventional camera on a Leitz Dialux 20 MB microscope (Leica Microsystems GmbH, Wetzlar, Germany), the images were digitized and the pollen tube lengths were measured using Metavue software (Universal Imaging Corporation, West Chester, PA).

Cytological analysis of pollen

Mature pollen grains were stained with 5 µg/ml fluorescein diacetate (FDA) in liquid pollen germination medium (as described in *'In vitro pollen germination and pollen tube growth'* but without agar) or with 4',6-diamidino-2-phenylindole staining solution (DAPI) as described by Park *et al.* (1998), and observed using epifluorescence microscopy. Germinating pollen, 1-1½ h incubated on semi-solid pollen germination medium, were stained for callose, cellulose or pectin using decolorized Aniline Blue (Polysciences, Warrington, Pa.), 0.001% Calcofluor White (Fluorescent Brightener 28; Sigma) or 0.01% Ruthidium Red (Sigma) in liquid pollen germination medium, respectively, and analyzed using a

fluorescence Leitz Orthoplan (Leica Microsystems GmbH, Wetzlar, Germany) microscope, equipped with a Colour Coolsnap digital camera (Roper Scientific, Tucson, AZ).

Cryo-scanning electron microscopy

Fresh pollen were rapidly frozen in nitrogen slush and then transferred to the vacuum chamber of the Cryo-Field-emission Scanning Electron Microscope (SEM, Jeol-JSM 6330F). After 4 min sublimation at -90°C, the samples were sputtered with gold/platinum for 40 s at -140°C, and examined in the microscope at -140°C.

In vivo pollen tube growth analysis by aniline blue and β-Glucuronidase (GUS) staining

Closed Col-0 flower buds were emasculated and these pistils, or pistils from mature flowers of *ms1* plants, were manually pollinated fully, or with exactly 20 pollen grains. The pollen tubes within the pistil were visualized by Aniline Blue staining as described in Kandasamy *et al.,* 1994. The pollinated pistils were fixed in ethanol:acetic acid (3:1) for approximately 30 minutes and softened in 1 N NaOH overnight. The samples were washed in distilled water, stained with decolorized Aniline Blue, squashed and examined with a conventional fluorescence microscope (Leitz Orthoplan/Ortholux combination) equipped with the appropriate illumination and filters for excitation and emission.

To analyze mutant pollen tube growth in fully competitive conditions, *bup-1/+*was crossed with the LAT52-GUS transformants. Mature pistils were pollinated with exactly 20 pollen grains of heterozygous *bup-1/+* plants homozygous for the LAT52-GUS construct, and subsequently were copiously pollinated with wild-type pollen. The bup-1/+ GUS/GUS pollen were histochemical stained for GUS activity, as described by Jefferson *et al.* (1987). The pistils were for 15 minutes in 90% acetone at 0°C, followed by three washes in 1M phosphate buffer (pH 7.2) for 15 minutes at room temperature. All tissues were stained in 0.1 M phosphate buffer (pH 7.2), 10 mM EDTA, 0.2% triton X-100, 2mM K3Fe(CN)6, 2mM K4Fe(CN)6, 1 or 2 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylamine salt (X-gluc) at 37°C. The pistils were decolorized in 70% ethanol at 4°C, and the GUS-staining pollen tubes visualised using a microscope.

Transmission electron microscopy

The transmission electron microscopy procedure was essentially as described previously (Derksen *et al.,* 2000). In short, membranes with pollen tubes grown for about 1-2 h were plunged into liquid propane 3.5 (Air Liquide, Belgium; –175°C), transferred into liquid nitrogen and freeze substituted in a Reichert-Jung (Austria) freeze-substitution apparatus. After substitution at –90°C for 36 h in 1% OsO_4 and 0.1% uranyl acetate in acetone, the temperature was slowly raised (4°C/h) to room temperature. Membranes with pollen tubes were embedded in Spurr's resin (Agar Scientific) and mounted on Sylon-CT-coated (Supelco, Bellefonte, Pa.) slides. The dialysis membrane was removed during the trimming process. Longitudinal sections were made with a MT5000 micro-tome (Sorvall, Newton, Conn.), post-stained with uranyl acetate and lead citrate according standard procedures, and examined in a 100CX II transmission electron microscope (Jeol, Tokyo, Japan).

Results

The male gametophytic mutants *bup-1/+* and *bup-2/+* are defective in pollen development or pollen tube growth. Hemizygous gametophytic mutants produce 50% wild-type pollen, which could complicate the statistical and cytological analyzes of the mutant phenotype. Therefore, *bup-1/+* was crossed with the sporophytic *quartet 1* (*qrt1*) mutant, in which the four products of meiosis stay attached in a tetrad structure during pollen maturation (Preuss *et al.,* 1994). In the *bup-1/+ qrt1/qrt1* mutant, two members of the tetrad carry the T-DNA induced mutation in *BUP*, while the other two are wild-type pollen. To study at which stage reproduction is affected, we analyzed pollen development, pollen hydration, germination and the progamic phase in these mutants by light- and electron microscopy.

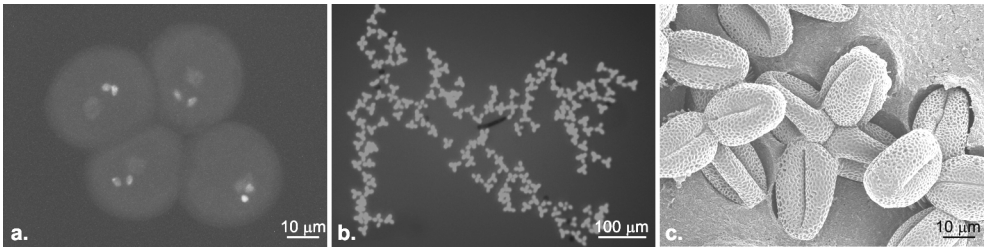


Figure 1: *bup* pollen development analysis in *bup-1/+ qrt1/qrt1* tetrads
a. DAPI staining showed a normal positioning of the vegetative cell nucleus and the two brightly stained sperm cell nuclei. b. FDA staining of ~100 tetrads showed that all the four pollen grains in the *bup-1/+* tetrads were viable. c. Cryo scanning electron micrograph showing four normally dehydrated pollen with normal exine architecture. Colour figure on page 170.

Pollen development and dehydration

Pollen development and pollen viability of line *bup-1/+* was tested by staining mature pollen with 4',6-diamidino-2-phenylindole (DAPI) and with fluorescein diacetate (FDA). DAPI stains DNA, and mature *bup-1/+ qrt1/qrt* tetrads showed normal positioning of the three nuclei in all four pollen grains (figure 1a). The vegetative nucleus was positioned in the centre of the vegetative cell, and the more intensively stained nuclei of the sperm cells were positioned next to the vegetative nucleus, as in wild-type pollen. The mitotic nuclear divisions and cytokinesis of the microspores, resulting in the production of the two sperm cells and the vegetative cell, thus occurred normally. FDA is a test for general esterase activity and plasma membrane integrity, and is considered a general viability stain for mature pollen (Heslop-Harrison and Heslop-Harrison, 1970). As is shown in figure 1b, the majority of the *bup-1/+ qrt1/qrt* tetrads showed four brightly stained pollen, as in wild-type tetrads, indicating that the pollen contained an intact plasma membrane and were still alive at this mature stage. The allelic *bup-2/+* mutant showed similar results in these tests, both with DAPI and FDA (data not shown). In addition,

scanning electron microscopic analysis showed that the mature *bup-1/+* and *bup-2/+* pollen appeared normally dehydrated upon anther dehiscence and no alterations in the pollen architecture and exine composition was visible (figure 1c). Pollen development and maturation thus appears not to be affected in these mutants.

Therefore, the gametophytic mutations *bup-1/+* and *bup-2/+* must become manifest during germination, during pollen tube growth or during the fertilization process itself. Upon self-pollination, the siliques of *bup-1/+* and *bup-2/+* were filled with mature seeds, indicating that most ovules were fertilized. Hypothetically, if the mutant pollen tubes would penetrate the embryo sacs but would not be able to realize fertilization this would result in empty places within the siliques. Since seed set in the mutants was normal, mutant pollen tubes, if formed, presumably do not reach the ovules. Thus, as both pollen development and fertilization appeared to be normal, we concluded that the gametophytic mutations *bup-1/+* and *bup-2/+* act during pollen hydration or germination, pollen tube initiation or during the progamic phase of sexual reproduction.

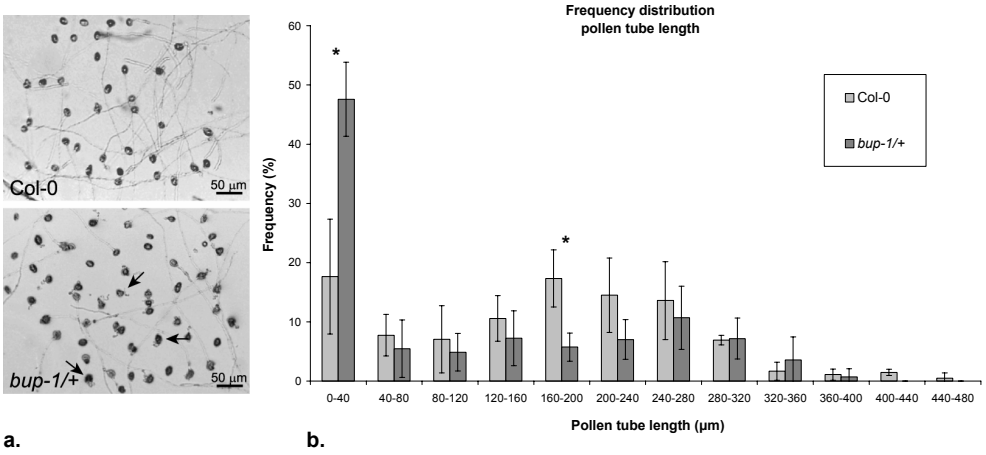


Figure 2: pollen tube length analysis
a. *In vitro* pollen tube growth after overnight incubation. *bup-1/+* contains short pollen tubes or pollen tube-like structures (arrows) which are not present in Col-0 b. Representative frequency distribution graph of pollen tube lengths after overnight *in vitro* pollen tube growth. Bars represent standard deviations of four bup-1/+ and three Col-O pollen populations tested in this particular experiment. Total number of pollen tubes *bup-1/+* n=220, Col-0 n= 232, * t-test $p<0.05$.

Pollen hydration, pollen germination and pollen tube growth *in vitro*

Pollen germination and pollen tube growth were tested *in vitro* on a solid pollen germination medium as described by Derksen *et al.*, 2002 (Chapter 2). No defect in hydration of *bup-1/+* pollen was observed as they swelled within minutes after application to the germination medium, like wild-type pollen. After overnight incubation, however, a proportion of the hemizygous *bup-1/+* pollen tubes

appeared much shorter than the wild-type pollen tubes, as short pollen tube-like structures were visible near the pollen grains (figure 2a, arrows). Frequency distribution analysis of the length of the pollen tubes showed that a significant large proportion of the *bup-1/+* pollen tubes were shorter than 40 µm (figure 2b), while wild-type pollen tubes showed a normal distribution in length. This difference in pollen tube length was already visible after 2 to 4 hours incubation (data not shown), though it was most obvious after incubation overnight, when more pollen were germinated and had started pollen tube growth. Tetrad analysis of pollen tube growth using the *bup-1/+ qrt1/qrt1* mutant confirmed the significant difference between the proportion of normal pollen tubes and that of the short pollen tube-like structures (table 1). Whereas wild-type *qrt1/qrt1* tetrads often had two, three or four tubes per tetrad (respectively 35%, 15% and 2%), this was rarely seen in the *bup-1/+ qrt/qrt* tetrads, of which only 15% had two pollen tubes, 1% had three pollen tubes and no tetrad with four pollen tubes was ever found. In addition, the frequency of tetrads lacking normal pollen tubes but with one or more of the short pollen tube-like structures was significantly higher in the *bup-1/+ qrt1/qrt1* mutant (37%) than in the *qrt1/qrt1* plants (15%) (table 1).

Table 1: Tetrad analysis of *bup-1/+* pollen tube growth *in vitro*

Plant genotype	# tetrads [a]	% Only short tubes	% 1 tube	% 2 tubes	% 3 tubes	% 4 tubes
qrt1/qrt1	190	13.6	28.6	**32.6**	**14.6**	2.2
bup-1/+ qrt1/qrt1	113	**37.2**	47.3	14.6	0.8	0
t-test p =		**0.02***	0.12	**0.02***	**0.01***	0.08

[a] tetrads that had no germinated pollen were not included in the analysis, * $p<0.05$ significant difference

Microscopic analysis at a higher magnification showed that the short pollen tube-like structures of the mutant pollen were in fact not real pollen tubes but curled cytoplasmic remnants attached to the pollen grain (figure 3), indicating that either the *bup-1/+* pollen grain or the pollen tube had burst. Although this 'exploded' phenotype was also found in wild-type pollen, it was statistically less frequent than in the mutant. *bup-1/+* pollen tubes were stained with Aniline Blue and Calcofluor White to analyze the nature of the cytoplasmic protrusions. Aniline Blue and Calcofluor White stain callose and crystalline cell wall material like cellulose, respectively. Fluorescent staining of wild-type pollen tubes showed that both callose and cellulose were present in the pollen tube wall (Chapter 2), but no such staining was found in the cytoplasmic protrusions (data not shown). The absence of pollen wall material indicated that it was the pollen grain that had burst, and not the pollen tube.

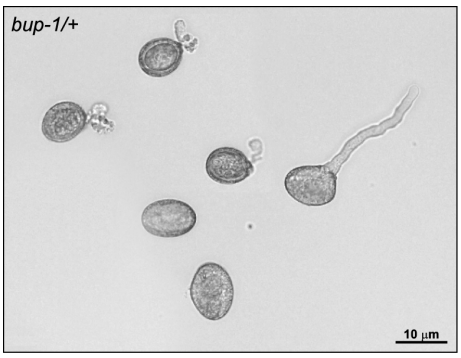


Figure 3: *bup-1/+* burst pollen phenotype
DIC microscopic analysis of *bup* pollen, showing the presence of curled cytoplasmic protrusions.

Cytochemical and ultrastructural analysis of pollen germination

During pollen germination, polarization of the pollen grain is marked by the deposition of callose at the site of pollen tube emergence (Johnson and McCormick, 2001; Lalanne *et al.*, 2004). This plaque of cell wall material not only stained with Aniline Blue, but also with Calcofluor White and Ruthidium Red, showing that also cellulose and pectin are present (figure 4). No difference in the position and accumulation of callose, cellulose or pectin during germination was shown between wild-type and *bup-1/+* and *bup-2/+* pollen.

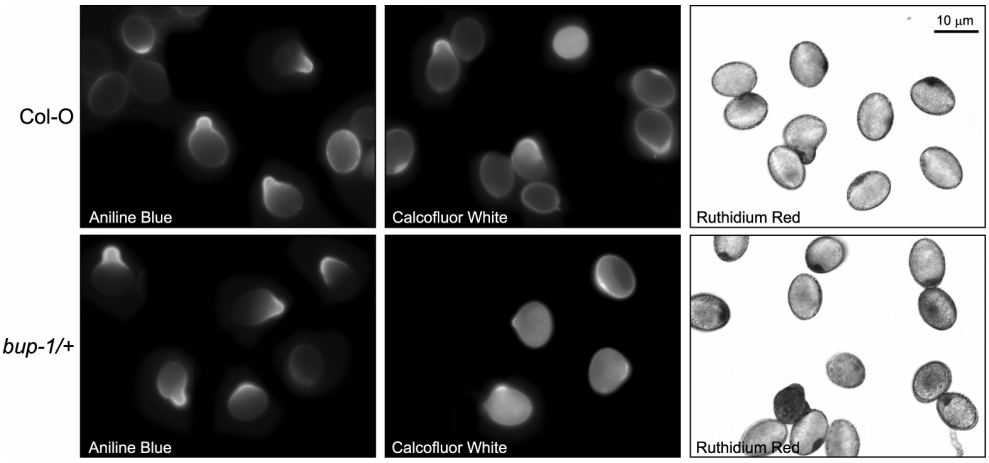
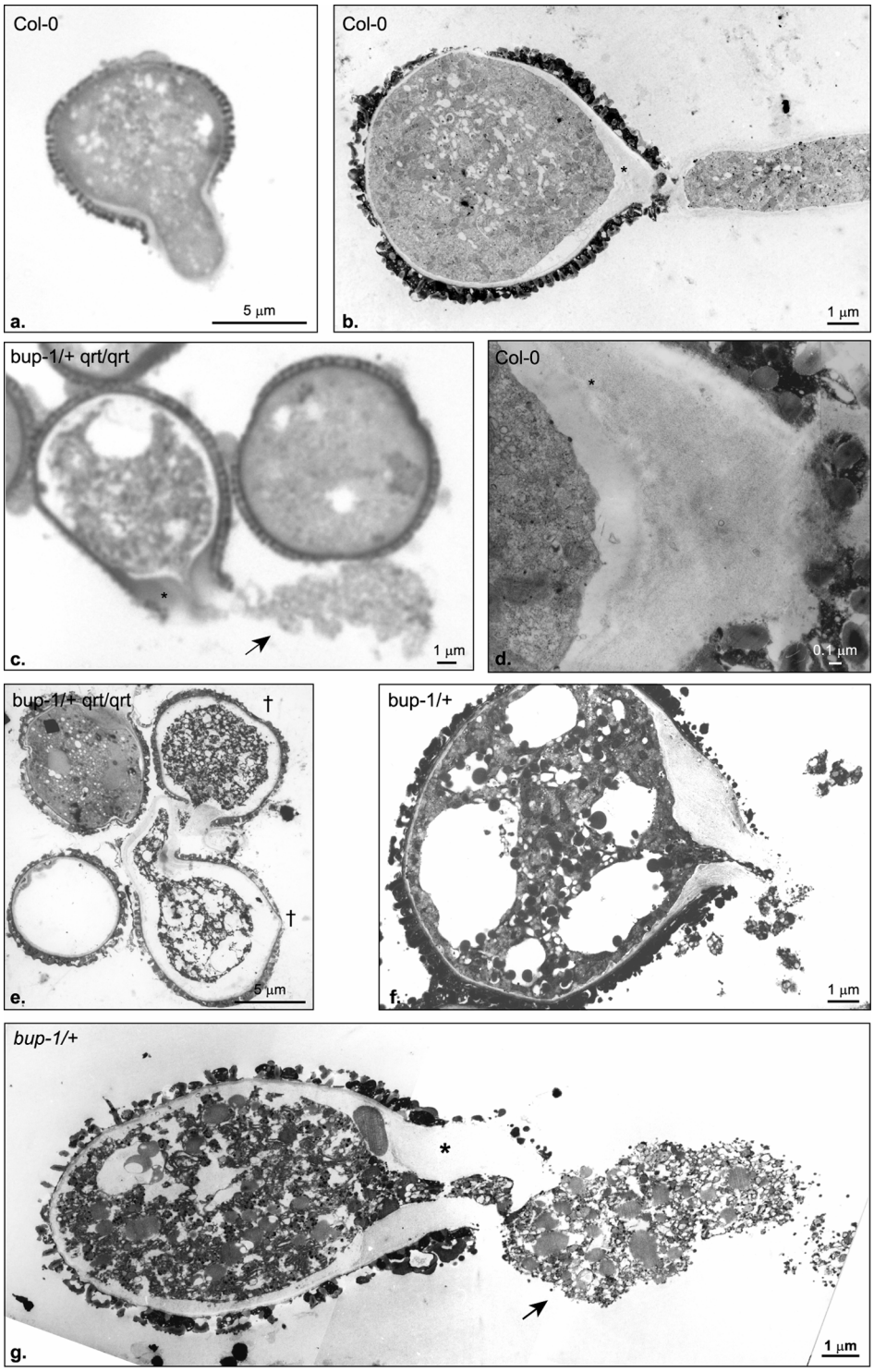


Figure 4: Germination plaque composition
Aniline Blue, Calcofluor White and Ruthidium Red staining of fresh germinating wild-type and *bup-1/+* pollen. Colour figure on page 170.

Next page: figure 5: Ultrastructural analysis of *bup-1/+* pollen
Toluidine Blue stained sections (a and c) and transmission electron micrographs (b and d-g) of Col-0, *bup-1/+* and *bup-1/+ qrt1/qrt1* germinated pollen. d, Magnification of plaque in wild type pollen in b. e, †: dead pollen in the tetrad. The other pollen grains have formed a pollen tube (empty pollen) or did not germinate. Arrows: Cytoplasmic protrusions of the mutant pollen, lacking a plasma membrane and cell wall material. Asterisk: germination plaque in wild-type and burst pollen. In the burst pollen, the cytoplasm seemed to have been pushed through a small opening within the plaque. Colour figure on page 171.


Col-0
a.
5 µm
Col-0
b.
1 µm
bup-1/+ qrt/qrt
c.
1 µm
Col-0
d.
0.1 µm
bup-1/+ qrt/qrt
e.
5 µm
bup-1/+
f.
1 µm
bup-1/+
g.
1 µm

For ultrastructural analysis, germinating wild-type (Col-0), *qrt1/qrt1*, *bup-1/+* and *bup-1/+ qrt1/qrt1* pollen (1½ - 3 hours incubation) were fixed chemically or by freeze substitution, embedded in plastic, sectioned and directly stained with Toluidine Blue or analyzed using transmission electron microscopy. In wild-type pollen, pollen tube initiation was started at the site of the plaque, by bulging. The pollen tube seemed to grow through the plaque, with the newly synthesized pollen tube wall having the same translucent electron-density as the plaque (figure 5a and b). Transmission electron microscopic observations of the *bup-1/+* and *bup-1/+ qrt1/qrt1* mutant pollen, one hour after germination, showed the presence of dead pollen, visible by the rough, electron-dense appearance of the cytoplasm and the plasma membrane that was detached from the pollen wall (figure 5d, f-g). Some of the pollen had germinated before they died, as shown by the deposition of the polarized plaque. However, instead of forming a pollen tube, the cytoplasm of these germinated pollen grains seemed to be forced out of the pollen grain, through a small opening within the plaque (figure 5c-g). No wall material or plasma membrane was shown to surround the cytoplasmic extrusions (figure 5c, e-g). The polarized plaque generally was smooth, slightly layered and seemed to be continuous with the intine layer, like in wild-type geminating pollen. Occasionally however, irregular fibrous structures or organelle inclusions could be observed in the plaque of the mutant pollen grains (not shown).

Taken together, we conclude that the mutant showed a range of pollen germination defects that might suggest a disturbance in pollen wall construction. The pollen either died before or during germination, in the latter case the cytoplasm burst out through the polarized plaque. This bursting of the germinating pollen could suggest a defect in the intine layer or the plaque, which therefore might not be able to hold the increased turgour during pollen hydration and germination.

Progamic phase and the fitness of the mutant pollen

Previous genetic studies showed that both *bup-1/+* and *bup-2/+* had a strongly reduced male transmission efficiency (TEmale=1%) of the T-DNA induced mutation to offspring (Chapter 3). The fact that a small proportion of the T-DNA mutated pollen can achieve fertilization indicated that the mutation may cause a quantitative defect in pollen fitness, as some pollen apparently were able to form a pollen tube under optimal conditions. To further analyze this, the effect of competition with wild-type pollen on male T-DNA transmission was tested. Mature wild-type pistils were fully pollinated or pollinated with exactly 20 *bup-1/+* pollen grains. Since every *Arabidopsis* ovary contains approximately 50 ovules, no competition between the pollen tubes was expected during fertilization upon limited pollination. Table 2 shows that the T-DNA transmission through *bup-1/+* pollen increased from 1.5% TE after full pollination to 15% TE after limited pollination. Thus, in the absence of competition with wild-type pollen the mutant pollen tubes had an increased

capacity to fertilize the egg cells and to produce viable seed. However, male transmission upon limited pollination was not fully restored to 50%, indicating that most of the mutated pollen grains were not able to perform fertilization.

Table 2: *bup-1/+* male T-DNA transmission is sensitive to competition

Pollen donor	# seed	Kan^R	Kan^S	*TE male*	p (t-test)
Full pollination *bup-1/+*	1737	25	1708	**1.5%**	0.0021
Limited pollination *bup-1/+*	349	47	307	**15.0 %**	

Mature wild-type (Col-6(*gl1*), Col-0 or *ms1*) pistils were pollinated by hand with plenty of *bup-1/+* pollen (full pollination, 71 pistils) or with exactly 20 *bup-1/+* pollen grains (limited pollination, 47 pistils). TEmale = transmission efficiency of the T-DNA through mutant pollen to the offspring. KanR/KanS x100. $p<0.05$.

In vivo pollen tube growth

To analyze mutant pollen germination and pollen tube growth *in vivo*, emasculated Col-0 or Col-6*(gl1)* pistils or pistils of the *ms1* mutant were pollinated with *bup-1/+* pollen and stained with Aniline Blue one hour to one day after pollination. The large number of pollen tubes upon full pollination made it difficult to analyze individual pollen grains or pollen tubes and therefore we chose to pollinate the stigmas with a limited amount of pollen.

No difference between pollen tube growth on and in the pistil was observed between wild-type and mutant pollen tubes after limited pollination. Generally, fewer pollen than applied remained attached to the stigma after staining and pollen that had not grown a pollen tube could have been lost during the staining procedure. Pollen tubes of both *bup-1/+* and wild-type pollen showed unexpected growth directions within the pistil. Limited pollination with wild-type pollen resulted in pollen tubes that, one day after pollination, had grown up and down the septum surface, seemingly without a clear direction of growth and sometimes even bypassing available ovules. This was not the case after full pollination when pollen tubes were in close vicinity and were forced to grow unilaterally to the ovules. The lack of directional pollen tube growth after limited pollination, however, made it very difficult to distinguish mutant pollen tubes. The limited pollination reduces the competition with wild-type pollen, and if only the speed of pollen tube growth was impaired, it would be difficult to distinguish mutant from wild-type pollen tubes.

Thus, although limited pollination might facilitate the visualization of single pollen tubes, competition in full pollinations is needed to analyze the effect of the *bup* mutation *in planta*. In order to set up an experiment with a limited amount of *bup-1/+* pollen tubes in full pollination, *bup-1/+* was crossed with a *LAT52::GUS* transgenic line, and *bup-1/+* plants homozygous for the *LAT52::GUS* construct were selected from the F2 generation. A limited amount of *bup-1*/+ *GUS/GUS*

pollen was applied onto the mature stigma, after which the pistil was fully pollinated with Col-0 pollen. One day after pollination the pistils were fixed and stained for GUS. Yet, with this method no clear mutant phenotype of the blue-stained pollen tubes could be distinguished. Like with Aniline Blue staining, fewer pollen grains than applied appeared to be present after staining for GUS activity, indicating that some of the GUS pollen were displaced from the stigma during the procedure. This apparent defect in pollen adherence *in vivo* agrees with the pollen germination defect found *in vitro*.

Screening for *bup-1* homozygous mutant plants

A major disadvantage of analyzing a male gametophytic mutant is that it only exists as hemizygote when the mutation is fully penetrant. Wild-type pollen will always be present, which hampers detailed cytological analysis of a quantitative or subtle pollen phenotype. The mutation of *BUP* however, is not fully penetrant and 1% of the T-DNA carrying pollen still can contribute to fertilization in lines *bup-1/+* and *bup-2*, indicating that fertilization of a T-DNA carrying egg cell by a T-DNA carrying sperm cell is possible. In theory, 0.5% (i.e. 1% male transmission x 50% female transmission) of the embryos upon self pollination should be homozygous for the mutation. However, while analyzing several thousand plants of different generations of *bup-1/+* and *bup-2/+* for segregation distortion (Chapter 3), no plants with undeveloped (empty) siliques, the expected phenotype of a homozygous male gametophytic mutant, had been found. To more actively search for homozygous mutants, *bup-1* mutants were analyzed by PCR for a homozygous T-DNA insertion, but no homozygotes were found (see the Appendix). Two other experiments, 1. a genetic screen using T-DNA insertional *BUP* alleles with a different selection marker crossed with *bup-1/+* and selection of the progeny for resistance to the two antibiotics, and 2. forcing the formation of sporophytic homozygous mutants, by pollen-specific complementation of the *bup* mutation using the LAT52 pollen-specific promoter of tomato, were attempted but gave no results. A description of these experiments can also be found in the Appendix.

Discussion

bup-1/+ mutant phenotype

Arabidopsis T-DNA insertional mutants of the At5g04480 gene that encodes for a glycosyl transferase protein, show a strong defect in male transmission of the T-DNA. While pollen development and fertilization appeared normal, *in vitro* pollen tube growth analysis showed a defect in pollen germination, indicated by the presence of cytoplasmic extrusions lacking a plasma membrane and cell wall. Wild-type *Arabidopsis* pollen germination is characterized by polarized accumulation of cell wall material at the site of pollen tube emergence. This 'plaque' contains callose, which has been used as a marker for pollen germination in several *Arabidopsis* pollen mutants (Johnson and McCormick, 2001; Lalanne *et al.*, 2004). Using Calcofluor White and Ruthidium Red we showed that this plaque also contains cellulose and pectin. The deposition of these cell wall components after hydration could function to regulate pollen tube emergence, by altering the strength of the pollen grain wall at this site. Ultrastructural analysis of germinating *bup-1/+* pollen showed quite a number of dead pollen that had died after hydration or during germination. These results indicated that a defect in the structure of the accumulated plaque or in the intine layer at the site of pollen tube emergence might be the reason for the bursting of the germinating pollen. No difference in the callose, cellulose and pectin localization was found. However, it is unlikely that a change in the composition of these compounds would be visible using these stains.

A defect in mutant pollen germination or during the initiation of pollen tube growth agrees with the fact that no mutant pollen tubes were observed *in vivo* after full and limited pollination using Aniline Blue and GUS staining. Though pollen adhesion to the *Arabidopsis* stigma was reported to be strong and occur directly after pollination (Zinkl *et al.*, 1999), the extensively treated pollinated pistils may have lost pollen grains that were not anchored to the stigma by a pollen tube.

Despite the significant defect in pollen germination *in vitro*, the gametophytic mutants still have 1% male T-DNA transmission to the offspring, indicating that some of the mutant pollen are successful in forming a pollen tube and can perform fertilization. These mutant pollen tubes however, also have a defect in growth. The ultrastructural analysis of the mutant pollen and the analysis of the pollen tube growth *in vivo* would be greatly facilitated by a homozygous mutant pollen population. Unfortunately, no homozygous mutant was identified in screens based on PCR- or phenotypic analysis. This difficulty in identifying homozygous mutants suggests that the homozygous mutation of gene At5g04480 maybe embryo lethal, as has been described for the cellulose synthase-like putative glycosyl transferase AtCSLA7 (Goubet *et al.*, 2003).

Putative role for BUP in the pollen germination and pollen tube growth

Glycosyl transferases of the GT1 family are transmembrane proteins with a single transmembrane domain, involved in the synthesis of carbohydrous cell wall components and glycoproteins. The pollen grain and pollen tubes contain many components that could be potential substrates, but so far only a few glycosyl transferases have been implicated to be involved in pollen functions. Putative substrates that BUP could act upon include the polyglycans that make up the pollen cell wall, and pollen glycoproteins and arabinogalactan proteins (AGPs). Alternatively, BUP might indirectly have a role in the synthesis of these components by the glycosylation of one of the enzymes involved.

The pollen cell wall consists of cellulose and callose, and the outer layer is largely made up of pectin (Schlüpmann *et al.*, 1994). As cellulose and callose are synthesized by the multiple membrane-spanning cellulose- and callose-synthases of the GT2 family (Richmond, 2000), BUP is not likely to be involved in their synthesis. Pectin however, is synthesized within the Golgi apparatus by polymerization of UDP-galacturonic acid monomers, followed by methyl-esterification of the homogalacturonan backbone residues and the addition of sugar residues as side chains. The highly methyl-esterified pectin is excreted by the Golgi-derived secretory vesicles (Gibeaut and Carpita, 1994). Pectin is an essential component within the pollen tube wall and perturbation of the pectin transport during pollen tube germination results in the arrest of pollen tube growth (Geitmann *et al.*, 1996). During pollen germination and pollen tube growth, an increased level of de-esterified pectin is present in the wall (Van Aelst and Van Went, 1992; Li *et al.*, 1992, 1994, 1995; Hasegawa *et al.*, 2000; Lennon and Lord, 2000; Suarez-Cervera *et al.*, 2002; Castells *et al.*, 2003; Abreu and Oliveira, 2004), indicating an increase in wall rigidity (Jarvis, 1984; Carpita and Gibeaut, 1993). This composition may be necessary to resist the increase in turgour pressure during pollen hydration and germination, and to strengthen the pollen tube wall during pollen tube growth. The *bursting pollen* phenotype might be caused by a defect in the structure of the intine layer or the plaque positioned at the site of pollen tube emergence. As BUP does not show homology to pectin synthases that synthesize the pectin backbones, like the pectin synthase in petunia pollen tubes (Akita *et al.*, 2002), BUP might be involved in the addition of the side chains that consist of many different sugar residues. Recently, an *Arabidopsis* mutant of the pectin methylesterase gene *VANGUARD1 (VGD1)* has been identified (Jiang *et al.*, 2005). *vgd1* mutants showed a severe reduction in pollen tube growth within the pistil, and *vgd1* pollen tubes burst *in vitro* (Jiang *et al.,* 2005), very much like the *bup* mutants do. The loss of VGD1 function was suggested to lead to loss or reduction of formation of Ca^{2+} pectate gel in the pollen tube wall, resulting in loss or reduction of strength of the pollen tube wall. Also the mutant phenotype of *bup* is

consistent with this theory, and this strengthens the hypothesis that BUP is involved in pectin metabolism in the pollen walls.

Besides the synthesis of polyglycans, glycosyl transferases are also involved in the glycosylation of proteins, resulting in glycoproteins and proteoglycans. Many glycoproteins are present within the male gametophyte, and the pattern of glycoproteins changes during pollen development (Říhová *et al.*, 1996, Hrubá *et al.*, 1999). Proteoglycans differ from glycoproteins by the large amount of glycosylation of the peptide backbone. A particularly interesting group of proteoglycans are the arabinogalactan proteins (AGPs), a highly glycosylated group of proteins that can contain over 90% (w/w) carbohydrate and can be membrane bound, wall-associated or soluble. The presence of AGPs near the intine layer in *Arabidopsis* pollen (Van Aelst and Van Went, 1992) suggests an important role during pollen germination. In some species, like *Lilium longiflorum* and *Annona cherimola,* AGPs are present at the tip of the pollen tubes, and treatment with the Yariv-reagent (that binds and precipitates AGPs) has shown that they are essential for pollen tube tip growth (Jauh and Lord, 1996; Roy *et al*, 1998; Mollet *et al*, 2002). A collar of AGPs and esterified pectin was shown to be present at the site of pollen tube emergence in pollen of lily, and AGPs therefore, might have a regulatory function in both pollen germination and pollen tube tip formation (Mollet *et al*., 2002; Abreu and Oliveira, 2004). In *Arabidopsis* however, like in *Lycopersicon pimpinellifolium*, *Aquilegia eximia* and *Nicotiana tabacum,* no AGPs seem to be present at the pollen tube tip (Mollet *et al*., 2002 and references therein). Nonetheless, *Arabidopsis* pollen tubes were killed by treatment with the Yariv-reagent (Roy *et al*., 1998; Lennon and Lord, 2000), and AGPs apparently do play a role in the function of *Arabidopsis* pollen tubes. The carbohydrate side chains of the protein backbones are very important for the function of both glycoproteins and AGPs. BUP, thus, could also be involved in the N- or O-glycosylation of pollen glycoproteins or it might be involved in the synthesis of AGPs important for pollen germination.

In conclusion, glycosyl transferases can be involved in the synthesis of various pollen or pistil-expressed components during the process of sexual reproduction, like pectin, glycoproteins and AGPs. The male gametophytic mutant *bup* has a defect during pollen germination. Here, cell wall material accumulates at the site of pollen tube emergence. Pectin synthesis, pectin modification and the presence of AGPs are all associated with the intine layer, and a defective glycosylation step of any of these compounds could result in a decrease in intine rigidity. The result that no homozygous mutant plants were identified, despite the strong effort to do so, suggests that BUP might be important for cellular metabolism, and in particular the cell wall synthesis, in sporophytic tissues as well.

Appendix: Screening for homozygous *bup* mutants

To identify homozygous *bup* mutants for the analysis of a sporophytic phenotype, PCR analysis was performed to identify homozygous T-DNA insertions in the *bup* gene. In addition, two other experiments were attempted to identify or create a homozygous mutant, though these gave no results. The three experiments are described in detail in this Appendix.

PCR-based screen for the identification of a homozygous T-DNA insertion

A T-DNA border primer was used in combination with a gene-specific primer to identify the insertional mutation, while two gene-specific primers were used for the identification of the wild-type allele. PCR analysis of 480 resistant offspring of *bup-1/+* plants, selected from Kanamycin germination plates and transferred to soil, resulted in eight plants with a T-DNA-insertion PCR product but no wild-type gene PCR product, indicating that these plants might be homozygous for the T-DNA insertion. However, six of the eight plants set seed, and selection of the offspring on Kanamycin plates showed that the plants were heterozygous for the Kanamycin resistance gene. The other two putative homozygous plants did not set seed, which is also the expected phenotype of a homozygous male gametophytic mutant. However, before these plants could be crossed or phenotypically analyzed in greater detail, the plants died due to an aphid infestation.

The promising result of this limited seed set phenotype led us to set up a new screen for the identification of a homozygous mutant plant, based on phenotype. 1470 *bup-1/+* seeds were directly sown onto soil without selection for Kanamycin. This was less time consuming and, in addition, could avoid the loss of resistant seedlings seen upon transfer from selection medium to soil during the initial PCR-screen. Phenotypic screening for limited seed set resulted in twenty plants with empty siliques of the first flowers. Subsequent PCR-analysis of the T-DNA insertion showed that in one of these plants no PCR-product was formed using the gene-specific primers, while the combination with the T-DNA-specific primer did give a DNA fragment of the expected size. Again though, the plant started to set seed, and selection of the offspring showed that the plant was heterozygous, giving rise to sensitive and resistant seedlings. The confusing results of an absent wild-type fragment in the PCR analyzes might be due to the fact that tandem and reverse T-DNA repeats are present within the insertion site in At5g04480, though this does not explain why the wild-type gene cannot serve as template in some of the PCR-reactions.

Genetic screen using T-DNA insertional BUP alleles with a different selection marker than Kanamycin

This experimental setup made use of an independent allele of gene At5g04480 (*bup-x*) that had been generated by the insertional mutation of a T-DNA with a resistance gene other than the NPTII gene. If this allele would also show the segregation distortion phenotype like *bup-1/+* and *bup-2/+*, the line could be crossed with *bup-1*. Selection of the F1 generation for both selection markers simultaneously would select for resistant seedlings that have the *bup-1/ bup-x* genotype, and therefore would be phenotypically homozygous for the mutated gene. With this method it would have been easy to screen large amounts of seed and would facilitate the identification of homozygous mutants. Should the homozygous mutation cause embryo lethality, the data obtained would be sufficient for quantitative statistical analysis to proof the abortion of the 0.5% homozygous embryos. The Gabi-KAT T-DNA population is made by the transformation with a T-DNA carrying the Sulfadiazine resistance marker (Li *et al.*, 2003; Rosso *et al.*, 2003). Two lines, 465B01 and 419E12, were annotated to have a T-DNA inserted within gene At5g04480, but PCR analysis of the T-DNA flanking sequences by GABI-KAT could not confirm the insertion site. Since no other independent alleles with a selectable marker other than Kanamycin resistance were available we could not proceed with this genetic search for *bup* homozygous mutants.

Pollen-specific complementation, forcing the formation of sporophytic homozygous mutants.

Since T-DNA carrying *bup-1* and *bup-2/+* pollen tubes can fertilize egg cells of wild-type pistils (1% male T-DNA transmission), these pollen tubes could also fertilize T-DNA carrying egg cells, resulting in homozygous embryos. The fact that no homozygous plant has been found so far might imply that the homozygous T-DNA insertion is lethal for the developing embryo. The low frequency of putative homozygous embryos (only 0.5%) made it very difficult to prove this hypothesis by screening siliques for aborted embryos upon self fertilization. If the mutant pollen tubes could be forced to participate in fertilization, the number of homozygotes would be much greater and aborted embryos would be much easier to identify. The pollen-specific *LAT52* promoter provides us with the possibility to actually do this, by transforming *bup* plants with the complementation construct (as is described in Chapter 3), in which the endogenous *BUP* promoter is replaced by the *LAT52* promoter. This way, the complementation would only act within the pollen, thereby forcing T-DNA carrying sperm cells to fertilize T-DNA carrying egg cells. After fertilization, the *LAT52* promoter then would be switched off and 25% of homozygous embryos would be present within the silique. This number is large enough to screen for embryo abortion, and if the seeds would survive also the sporophytic tissues of the plant could be screened for a homozygous mutant

phenotype. (Obviously, no pollen mutant phenotype can be analyzed in these homozygotes, since the *LAT52* promoter driven wild-type *BUP* allele would be complementing the mutation.) Unfortunately however, it was not possible to synthesize the complementation construct combining the *LAT52* promoter with the At5g04480 transcribed region within the limited time available, and the experiment could not be concluded.

Chapter 5

Expression analysis of a small gene family coding for the glycosyl transferase 1 proteins BURSTING POLLEN (BUP) and BURSTING POLLEN-LIKE (BPL)

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Abstract

BURSTING POLLEN (BUP) encodes a putative family 1 glycosyl transferase and by mutant analysis has shown to be important for pollen germination and pollen tube growth. Only one other *Arabidopsis* protein shows homology to BUP (BURSTING POLLEN-LIKE, BPL, with 35% similarity), though the precise function of this protein is not known. To investigate the role of the two genes in pollen and in sporophytic tissues, gene expression analysis was performed by promoter-reporter gene fusion and northern blot analysis (for *BUP)* and by RT-PCR and micro-array analysis, using data from pollen micro-arrays (Honys and Twell, 2004) and from the Genevestigator database (for *BUP* and *BPL*). Unfortunately, the results of the *BUP* promoter fusion constructs were inconclusive, as the strong constitutive 35S promoter driving the selection marker appeared to interfere with the expression of the *BUP* promoter-driven visible markers. Transcript analysis showed that both *BUP* and *BPL* are expressed constitutively in sporophytic tissues, *BPL* at a higher level than *BUP*. Consistent with its function in pollen germination and pollen tube growth, *BUP* is also expressed in mature pollen, though at a very low level and below the threshold for micro-array analysis. In contrast, *BPL* was found to be expressed only during early stage in pollen development, and not in mature pollen. The low expression level of *BUP* and its putative role in the fitness of the sporophyte are discussed and compared to the expression data of other GT1 genes. Functional analysis of the expression data in the Genevestigator database suggest that both *BUP* and *BPL* may be involved in the synthesis of cell wall components, in particular the synthesis of pectin.

Introduction

Sexual reproduction in flowering plants involves transition from the sporophytic stage to the gametophytic stage via meiosis. In the male gametophyte, sperm cells are produced and transported by the pollen tube to the haploid female reproductive cells of the embryo sac, where double fertilization occurs resulting in diploid offspring and triploid endosperm. Although the main purpose of the meiotic division is to reduce the number of chromosomes prior to fertilization, this by no means implies that the gametophytic genes are inactive until fertilization occurs. In contrast, some of the genes expressed in the pollen vegetative cell show very high expression levels and are specific for the male gametophyte. The genes expressed in the vegetative pollen cell are necessary for proper pollen development and germination, pollen tube growth and guidance towards the egg cell, while genes expressed in the sperm cells are considered to be involved in gamete recognition and early fertilization events (Russell, 1985; Xu *et al.*, 2002; Engel *et al.*, 2003; Da Costa *et al.*, 2003).

Genes expressed in pollen can be very broadly classified in two groups: the early expressed genes (genes that become active soon after meiosis is completed) and the late expressed genes (genes that become active after the first or second microspore mitosis; Mascarenhas, 1990). The first studies on quantitative gene expression in pollen were based on mRNA hybridization kinetics and isozyme analysis of pollen and sporophytic tissues. In various species, these studies showed a 60% overlap of genes expressed in both gametophytic and sporophytic tissues, while approximately 10% of the transcripts or isozymes were found specifically in pollen (reviewed in Mascarenhas, 1990). Recent pollen transcriptome micro-array experiments in *Arabidopsis* strongly support these data (Honys and Twell, 2003, 2004; Becker *et al.*, 2003). The most complete analysis used the Affymetrix ATH1 array hybridized with RNA of pollen at various developmental stages (Honys and Twell, 2004). This analysis showed that ~62% of the genes were expressed during pollen development, with ~90% showing pollen-sporophytic overlap and 9.7% specifically expressed in pollen. The RNA pool in mature pollen showed a very divergent expression profile, which was clearly different from the sporophytic transcriptome (Honys and Twell, 2003, 2004; Becker *et al.*, 2003). This difference seems to be caused mainly by the genes expressed after pollen mitosis. These ‘late’ pollen genes generally are expressed specifically in pollen and at a very high expression level, while most ‘early’ pollen genes are also expressed in sporophytic tissues and at a much lower level (Honys and Twell, 2004). The pollen-specific genes are preferentially involved in cell wall metabolism, signalling and cytoskeleton organization; genes related to photosynthesis or translation are underrepresented (Honys and Twell, 2003, Becker *et al.*, 2003; Lee *et al.*, 2003, Da Costa *et al.*, 2003). In contrast, the distribution of pollen-expressed

genes that were also expressed in the sporophyte followed the distribution of the sporophytically expressed genes (Honys and Twell, 2003). This indicates that within pollen, genes are expressed that qualitatively represent the sporophytic transcriptome.

Many *Arabidopsis* male gametophytic mutants have been identified so far (reviewed in McCormick *et al.*, 2004), though only a few have been characterized at the gene level. Mutants from T-DNA tagged populations have the advantage that the mutated gene can be isolated relatively easily. By TAIL-PCR, we were able to identify the mutated gene At5g04480 in a male gametophytic mutant that showed severe segregation distortion of the T-DNA (Chapter 3). This gene encodes for BURSTING POLLEN (BUP), a glycosyl transferase protein of the GT1 family (Egelund *et al.*, 2004). *bup* mutants are impaired in pollen germination and pollen tube growth (Chapter 4). Only one *Arabidopsis* protein is homologous to BUP (BUP-like (BPL) with 34% similarity), and this protein could be functionally redundant to BUP (Chapter 3).

Here we present the results of gene expression analysis of *BUP* and *BPL*, by means of promoter fusion analysis, northern blot and RT-PCR analyses, and we compare these results with the *'in silico'* expression data of micro-array analyses published (Honys and Twell, 2004; Zimmermann *et al.*, 2004) and available online.

Material and Methods

Plant Material

Seeds of *Arabidopsis thaliana* L. ecotype Col-O were obtained from the Nottingham *Arabidopsis* stock centre (NASC). *Arabidopsis* plants were grown in a 3:1 mixture of soil and vermiculite and kept in a growth chamber with a 16 hours photoperiod, at 22°C and ambient relative humidity. Pollen tubes for GUS staining were grown *in vitro* on pollen germination medium, as described in Derksen *et al.* (2002) and in Chapter 2.

Construction of promoter fusions and plant transformation

pCAMBIA 1302 and 1303 binary vectors were used as *35S::GFP* and *35S::GUS:GFP* constructs (Roberts *et al.*). The *BUP* promoter fusion was constructed as follows: a 2.3 kb fragment of the 5' flanking region of the *BUP* gene was obtained from BAC AtT32M21 DNA by high fidelity PCR using 25 pmol of the primer 5'- TGCTG**GGATCC**ACGGAGGGAACTGAGTTTGGAGGAGG-3' that has a BamHI restriction site included (bold letters), and 5'-GAGAGTTGTCGA**CCATGG**AGAAAGAAGAAAAACTGCT-CTGC -3' that contained the original NcoI site (bold letters), in 50 µl using 50 ng BAC DNA as template, 5 µl 10x Pfu reaction buffer, 2 µl 25 mM dNTP, 25 pmol of each primer and 1.5 units of Pfu DNA polymerase (Promega). The PCR program was as follows: 2 min at 95°C, 2 cycles of 15 s at 94°C, 30 s at 50°C, and 3 min at 72°C, followed by 25 cycles of 1 min 95°C, 30 s 58°C and 3 min 72°C and finalized with 10 min 72°C. The PCR product was ligated into pGEM-Teasy (Promega) and transformed into *Escherichia coli* strain DH5α. Plasmid DNA was isolated using standard alkaline lysis methods. The BamHI and NcoI *BUP* promoter fragment was cloned upstream of the GUS coding region in the vector pCAMBIA1302 or pCAMBIA 1303 by replacing the BamHI-NcoI *35S* promoter fragment, resulting in construct *BUP::GFP* and *BUP::GUS:GFP*, respectively. *LAT52::GFP and LAT52::GUS:GFP* constructs were made by directionally ligating the *LAT52* promoter SalI and NcoI fragment of pLAT52.7 into pCAMBIA 1302 or 1303, replacing the SalI-NcoI *35S* promoter fragment upstream of the reporter genes. Promoterless constructs were made by digesting these plasmids with BamHI and NcoI, filling of the 5' extruding ends with Klenow polymerase (Promega) and subsequent re-ligation of the plasmid backbone (figure 1). The binary vector constructs were transferred to *Agrobacterium tumefaciens* strain GV3101 using freeze-thaw transformation (Chen *et al.*, 1994). *Arabidopsis* plants were transformed by the floral dip method, as described by Clough and Bent (1998) and described in Chapter 3. T1 seeds were grown on agar plates containing 2.2 g/l MS salts, 0.5 g/l MES, pH 5.7, 7 g/l select agar, pH 5.8 and 20 mg/l Hygromycin. Resistant seedlings were transplanted to soil. The *LAT52::GUS* transformants using the pPZP221 plasmid were generated by Dr. Eric Lalanne (University of Leicester, England).

β-Glucuronidase (GUS) activity assay

Histochemical staining for GUS activity of the T1 and T2 generation of the promoter-fusion transformants was performed as described by Jefferson *et al.* (1987). Prior to staining, all material except the *in vitro* grown pollen tubes was fixed for 15 minutes in 90% acetone at 0°C, followed by three washes in 1M phosphate buffer (pH 7.2) for 20 minutes at room temperature. All tissues were stained in 0.1 M phosphate buffer (pH 7.2), 10 mM EDTA, 0.2% triton X-100, 2mM K3Fe(CN)6, 2mM K4Fe(CN)6, 1 or 2 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylamine salt (X-gluc) at 37°C. The pollen tubes were visualized directly using a light microscope (Zeiss axiovert 135 TV), the other tissues were stored in 70% ethanol at 4°C. Seedlings, whole flowers and siliques were visualized using the dissecting microscope (Zeiss, Oberkocken, Germany). Digital photographs were taken with a mounted CCD camera and the images were enhanced using Adobe Photoshop 5.0.

Northern blot analysis

Total RNA was isolated from rosette leaves and flowers by grinding the tissue in 1 ml extraction buffer (0.1 M Tris, 50 mM EDTA, 1% (w/v) SDS, 0.1 M NaCl, 1% tri-iso-propanylnaphtalene sulfonic acid sodium salt, 50 mM β-mercaptoethanol, pH 8.0) and 1 ml phenol. After phenol:chloroform

extraction and ethanol precipitation, RNA was obtained from the dissolved ethanol-precipitated pellet by LiCl precipitation. 10-20 µg total RNA was separated on 1.3% (w/v) agarose gels containing 0.5% (w/v) formaldehyde in MOPS buffer (Sambrook *et al.,* 1989). The RNA was then blotted onto a nylan membrane (Nitran supercharge) by capillary transfer in 10x SSC, and fixed by baking at 80°C for two hours. The blot was pre-incubated in hybridization buffer (6x SSC, 0.5% (w/v) SDS, 5x Denhardts solution and 0.1 mg/ml denatured herring sperm DNA). The *BUP* RT-PCR cDNA product was used as probe (see 'RT-PCR analysis' for primer sequences and PCR conditions). 25 ng DNA probe was ^{32}P-labeled by random primed labelling (Feinberg and Vogelstein, 1983) and hybridized to the RNA blot in hybridization buffer, overnight at 60°C. Also at 60°C, the filter was washed to 0.2xSSC/0.1% (w/v) SDS, and Kodak X-omat AR Scientific Imaging Films with intensifying screens or a phosphoimager screen (BioRad) were exposed to the blot at -80°C. All autoradiograms were digitized and the contrast and brightness were adjusted with Adobe Photoshop.

RT-PCR analysis

Total RNA was extracted from pollen, flower, leaf and stem tissue using Trizol® reagent, as described by the manufacturer (Life Technologies, Rockville, MD). For the isolation of mature pollen, inflorescences from over 100 plants were harvested in a large Erlenmeyer flask, 300 ml of ice-cold 0.3 M mannitol was added, and the flask was vigorously shaken for 1 min. The pollen suspension was filtered through a 53-µm nylon mesh. Pollen grains were concentrated by repeated centrifugation steps (50 ml Falcon tubes, 450 g, 5 min, 4°C), and the final compact pollen pellet was stored at -80°C. The RT-PCR reaction was as described by Honys and Twell (2003). 1 µg total RNA of each sample was reverse transcribed in a 20 µl reaction using the ImProm-II Reverse Transcription System (Promega, Madison, WI) following the manufacturer's instructions with the exception that the oligo(dT)15 primer was replaced with a custom-synthesized 3'-RACE primer (5'-AAGCAGTGGTAACAACGCAGAGTAC-(T)30VN-3'). For PCR amplification, 1 µl of 10x or 40x diluted RT mix was used. The PCR reaction was carried out in 25 µl with 0.5 units of *BioTaq* DNA polymerase (Bioline, London), 1.2 mM $MgCl_2$, and 10 pmol of each primer. The PCR program was as follows: 2 min at 95°C, 30 or 45 cycles of 15 s at 94°C, 15 s at 56°C, and 1 min at 72°C, followed by 10 min at 72°C. The conditions were 1.25 ng cDNA and 30 cycles for the Rubisco gene, 1.25 ng cDNA and 45 cycles for *BUP* and *APG-like*, and 5 ng cDNA and 45 cycles for *BUP-like* and the KAPP gene. Intron-spanning primers for *BUP* (At5g04480) 5'-CTCGTAAACGCCTTTCCAGTGT-3' and 5'-CTTCTGGACAAGATTAGACATCGATG-3', *BPL* (At4 g01210) 5'-CAGTTTCGTTCTCAGGCGGTG-3' and 5'-TTTGGTCGTTCTGTGATGCCA-3', *APG-like* (At4g28780) and KAPP (At5g19280) 5'- ATGGCGATGATAGGGATG-3' and 5'-GTAAAACCGTCCC-TCAGTC-3'. For Rubisco (At5g38430), the gene-specific primer 5'-ATCTTACCTCCCTGACCTTACT-GACGT- 3' was used in combination with a NESTED primer (5'-AAGCAGTGGTAACAACGCAGAGT-3') overlapping the 3'-RACE primer to eliminate genomic DNA amplification.

'In silico' expression analysis

Expression profiles of *BUP* and *BPL* were analysed using the Genevestigator database (*https://www.genevestigator.ethz.ch/*, Zimmermann *et al.,* 2004). That comprises all Affymetrix AG array (8K) and Affymetrix ATH1 (23K) array slides of the Nottingham *Arabidopsis* Stock Centre Transcriptomics Service (NASCArrays; Craigon *et al.*, 2004), ArrayExpress at the European Bioinformatics Institute (EBI; Brazma *et al.*, 2003) and the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI; Edgar *et al.*, 2002). *BUP* (At5g04480) and *BPL* (At4g01210) are represented on the ATH1 array, not the AG array. *BUP* and *BPL* gene expression patterns in different tissues or during development were analysed with 'Gene Atlas' and 'Gene Chronologer' respectively, using all 1434 Affymetrix ATH1 array slides present in the database. The percentage of present/absent calls for both genes within these slides was analysed using 'Digital Northern', with 'present' being significantly above background $p<0.06$. The scatter-plot was made with 'Gene Correlator'. Pollen expression of *BUP*, *BPL* and the GT1 gene family was analysed using the data from Honys and Twell (2004), supplement file 1 of the Genome Biology online publication. The

annotated GT1 genes were taken from the TAIR homepage (*http://www.Arabidopsis.org/info/gene-family/Glycosyltransferase.html*).

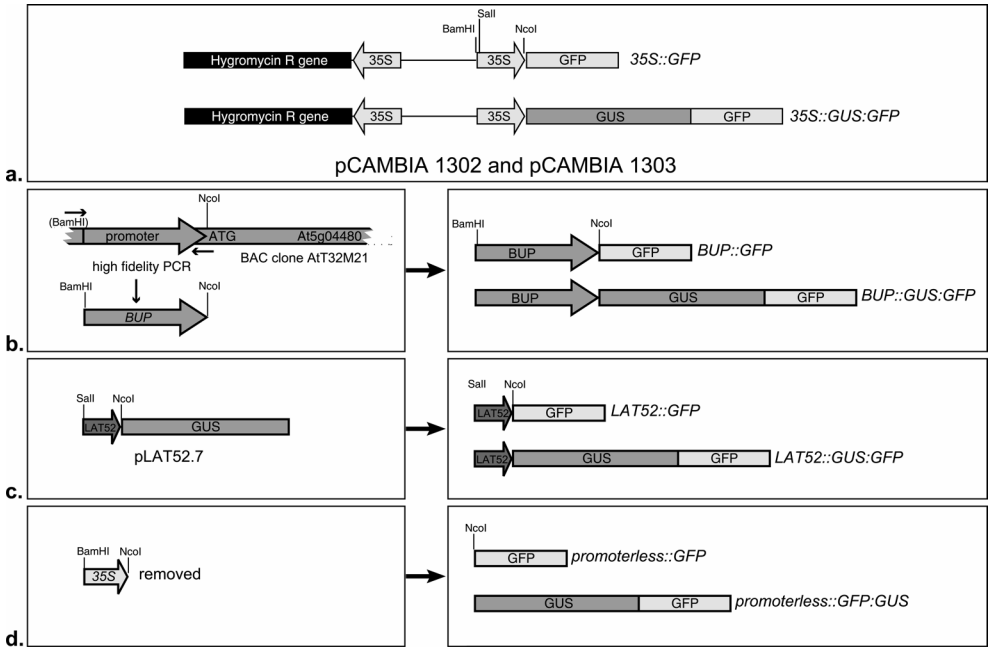

Figure 1: Promoter-*GFP* and promoter-*GUS:GFP* fusion constructs
The *35S* promoter in (a) the pCAMBIA 1302 and 1303 plasmids was replaced by (b) the *BUP* promoter, or (c) the *LAT52* promoter, or removed to create (d) the promoterless construct.

Results

Promoter fusion analysis

To analyse the spatial and temporal *BUP* expression pattern *in planta*, promoter fusions with the Green Fluorescent Protein (GFP) and the ß-Glucuronidase (GUS) genes were made, using the pCAMBIA 1302 and 1303 Ti plasmids. The *BUP* promoter region used for these constructs was derived from the BAC clone T32M21 (figure 1). As the same promoter fragment was contained in a complementation construct that had successfully restored the *bup* mutant phenotype to wild type upon transformation (Chapter 3), this region was held to contain the functional promoter. The *Cauliflower Mosaic Virus 35S* (*35S*) promoter, driving the *GFP* or *GUS-GFP* reporter genes in pCAMBIA 1302 and 1303, was replaced with the *BUP* promoter or the pollen-specific *LAT52* promoter (figure 1b and c). Additionally, the *35S* promoter was simply removed resulting in a promoterless marker construct (figure 1d). *Arabidopsis* ecotype Columbia (Col-0) plants were transformed with the original pCAMBIA vectors as well as with these modified constructs.

Pollen and pollen tubes of *LAT52::GFP* transformants showed a clear GFP signal, while no signal higher than the autofluorescence of the pollen wall was observed in the *35S::GFP* and the promoterless *GFP* plants, and in pollen of untransformed Col-0 plants. The pollen and pollen tubes of *BUP::GFP* transformants did not show a visible GFP signal (data not shown). Presumably, the *BUP* promoter was too weak to give a visible GFP signal in pollen.

For gene expression analysis using the GUS reporter gene, offspring of 15 independent *BUP::GUS:GFP* transformants were chosen for histochemical staining of GUS activity. Though the intensity and the staining pattern varied slightly between the individual lines, the overall pattern in plants transformed with the same construct was similar. *BUP::GUS:GFP* plants showed GUS staining in seedlings, leaves, ovary and style of the flower. Also the vascular bundles of the petals, no GUS expression was found in pollen or in pollen tubes grown *in vivo* or *in vitro* (figure 2 and data not shown). Also the *LAT52::GUS:GFP* plants showed GUS gene expression in most other tissues in addition to the expected staining of pollen and pollen tubes. The overall GUS expression pattern under control of the *BUP* or *LAT52* promoter was similar to that of transgenic plants carrying the *35S::GUS:GFP* construct. This result strongly suggested that the *35S* promoter driving the plant selection marker in this T-DNA (figure 1) is interfering with the GUS gene expression. Analysis of *LAT52::GUS* expression pattern in transformants generated with two different Ti plasmids confirmed this hypothesis. Table 1 shows that the presence of a *35S* promoter on the T-DNA of different Ti-plasmids resulted in an overall GUS expression pattern in *LAT52::GUS* transformants, while a combination of the *LAT52::GUS* fusion with the *NOS*

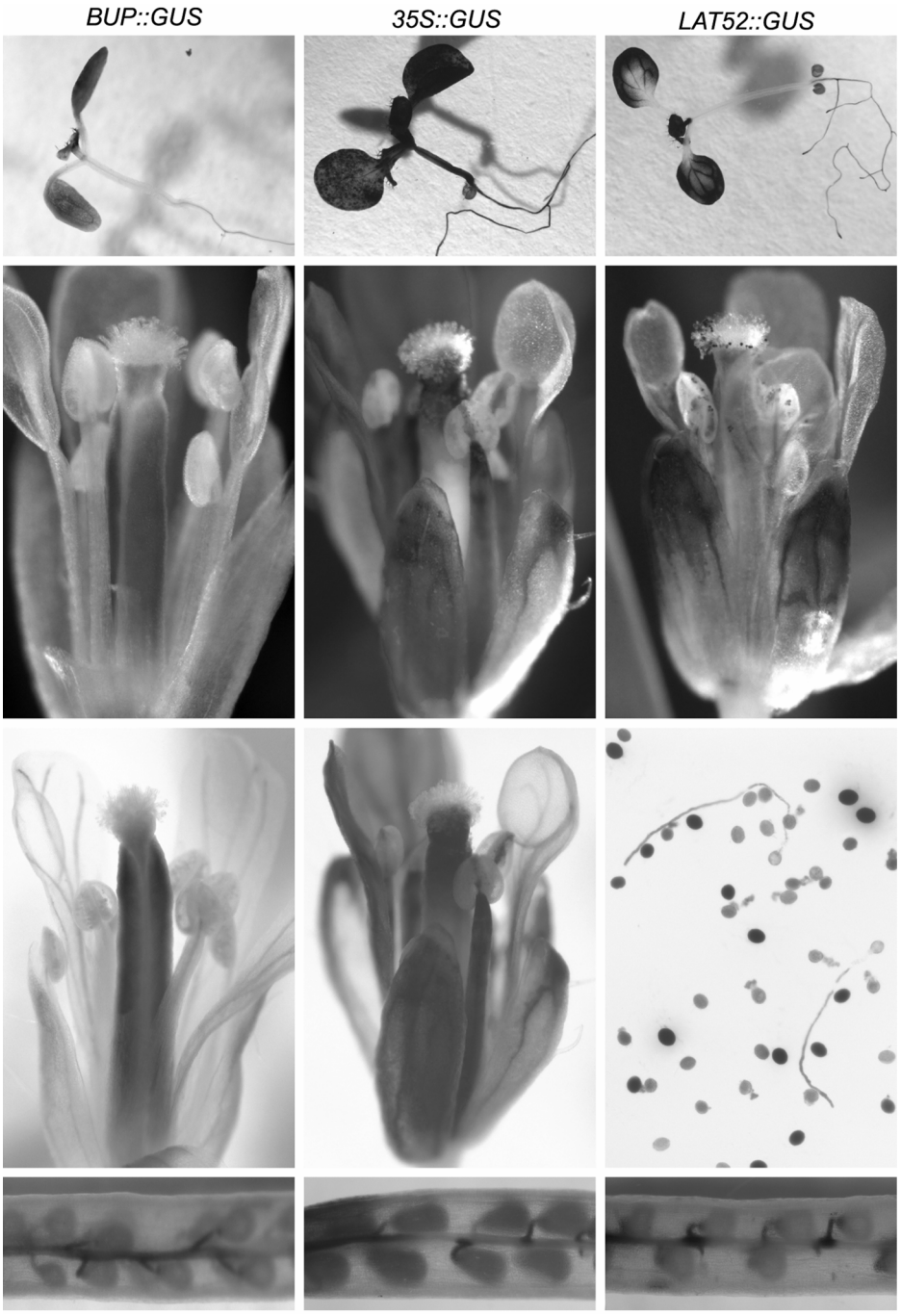

Figure 2: GUS histochemical localization of *BUP* expression
GUS staining of *BUP::GUS:GFP, 35S::GUS:GFP* and *LAT52::GUS:GFP* transformants showed similar expression patterns. As an exception, the pollen of *LAT52::GUS:GFP* transformants were also stained for GUS. Colour Figure on page 174.

promoter, resulted in pollen-specific expression of the *GUS* gene (Twell *et al.*, 1990). With the promoterless *GUS:GFP* construct, some independent transformants did not show blue staining in the GUS assay, but others did (data not shown). Analogous to the ectopic *LAT52::GUS* expression, this GUS gene expression might also be caused by the close vicinity of the *35S* promoter in the T-DNA of pCAMBIA 1303.

Table 1: T-DNA dependent GUS expression

Promoter driving GUS (Ti plasmid)	Promoter driving the plant selection marker	GUS expression and localization
BUP (pCAMBIA 1303)	*35S*	Overall (no pollen) [a]
35S (pCAMBIA 1303)	*35S*	Overall (no pollen) [a]
LAT52 (pCAMBIA 1303)	*35S*	Overall (including pollen) [a]
LAT52 (pPZP221)	*35S*	Overall (including pollen) [b]
LAT52 (pBIN)	*NOS*	Pollen-specific [b] (Twell *et al.,* 1990)

[a] shown in figure 2. [b] data not shown

Northern blot and RT-PCR analyses

Northern blot analysis using total RNA of flowers and leaves resulted in a weak signal for the *BUP* transcript in both tissues after several weeks of exposure (figure 3a). The hybridizing fragment was close in size to the 25S ribosomal RNA and to the predicted size of the *BUP* transcript (3.8 kb). RT-PCR analysis of *BUP* showed a signal in pollen, flower, leaf and stem samples. Although these data are not quantitative, the results suggest that the transcript was low abundant, as 45 cycles were needed to get a visible PCR product in pollen (figure 3b). *BPL* (gene At4g01210 coding for BPL that is homologous to the BUP protein) showed expression in sporophytic tissues, but no expression in mature pollen (figure 3b). The quality of the pollen sample was tested by RT-PCR analysis of the constitutively expressed *Kinase Associated Protein Phosphatase* gene (*KAPP*, At5g19280, Williams *et al.*, 1997), which resulted in a clear signal in pollen. The purity of the pollen sample was confirmed by the lack of expression of *APG-like* gene At4g28780 and of the Rubisco small subunit 1b gene At5g38430, both expressed in green tissues only (Kamada *et al.*, 2003; Honys and Twell, 2003).

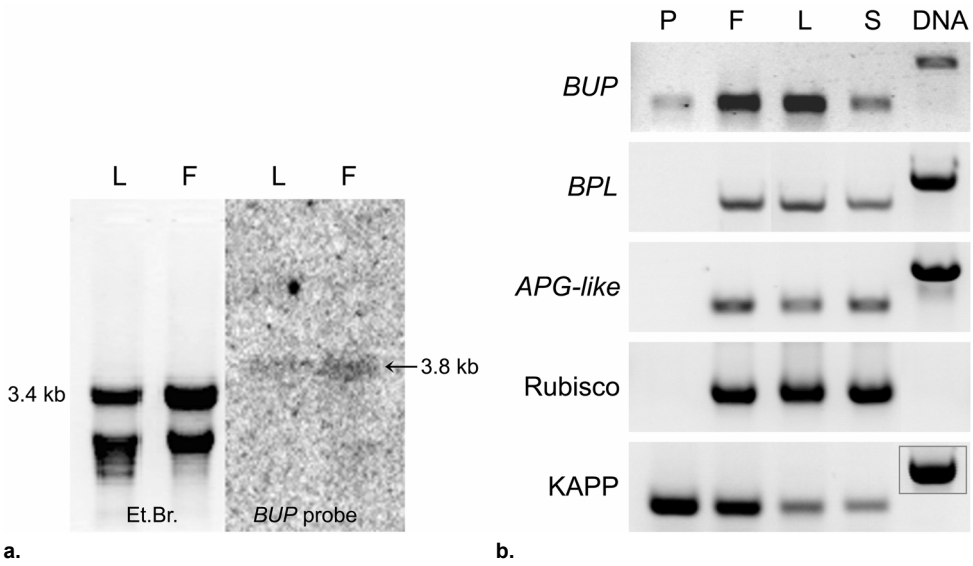

Figure 3: *BUP* and *BUP-like* expression analysis
a. Northern blot analysis using 20 µg of leaf (L) and flower (F) total RNA, hybridized with the *BUP* cDNA probe. Ethidium bromide (Et.Br.) staining shows equal sample loading. b. RT-PCR using pollen (P), flower (F), leaf (L) and stem (S) cDNA. A DNA control is present for *BUP*, *BPL*, *APG-like* and the *KAPP* gene (inset) for which intron-spanning primers were used.

In silico expression analysis of *BUP* and *BPL*

Analysis of the micro-array data present in the Genevestigator database (*https://www.gene-vestigator.ethz.ch/*) showed that *BUP* (gene At5g04480) was expressed at a low level, with a mean signal value of 500 probe-set-counts, in all sporophytic tissues and developmental stages (figure 4). In only 56% of the 1434 ATH1 array slides in the database, the gene is specified as 'present' ($p<0.06$), which also indicated that the gene had a very low expression level. The sporophytic expression of *BUP* was also confirmed by the presence of full-length cDNA sequences derived from leaves (AY052353, Shinn *et al.*, 2001) and siliques (BX833522, Castelli *et al.*, 2003) in GenBank (*http://www.ncbi.nlm.nih.gov*). *BPL* (gene At4g01210) expression analysis in Genevestigator showed that *BPL* was expressed in all tissues and developmental stages like *BUP*, but the signal value was 2-3 fold higher than that of *BUP* (figure 4a and b). In addition, *BPL* is called present in most slides of the database, and this difference is clearly visible in a scatter-plot showing the co-expression of *BUP* and *BPL* in the different ATH1 slides (figure 5a, all dark grey spots represent slides with only *BPL* present).
The highest expression levels of *BUP in silico* (black-encircled spots, figure 5a) were found in stem tissue and this was significantly higher than the signal values in flower and leaf in the same experiment (figure 5b, Osborne and Somerville, 2003). The highest expression levels of *BPL* (grey-encircled spots, figure 5a) were found

on slides of an experiment designed to analyse the expression of genes involved in the assembly of the cell wall matrix of *Arabidopsis* suspension-cultured cells. *BPL* expression in these cells was significantly increased upon treatment with the cellulose inhibitor isoxaben (figure 5b, Manfield *et al.*, 2004).

The expression of *BUP* and *BPL* in pollen could not be analysed using Genevestigator, as no specific annotation for pollen-expressed genes is present in this database. Therefore, we analysed the pollen transcriptome data generated by Honys and Twell (2004). No signal for *BUP* was present in any of the four successive pollen developmental stages (uninucleate microspores, bicellular pollen, tricellular pollen and mature pollen grains, table 2). *BPL* however, was expressed in the uninucleate microspores and bicelullar pollen, but not in pollen at later stages, indicating that this is an early pollen expressed gene (table 2). The expression in sporophytic tissues that were included in the pollen transcriptome analysis confirmed that both *BUP* and *BPL* are also expressed sporophytically.

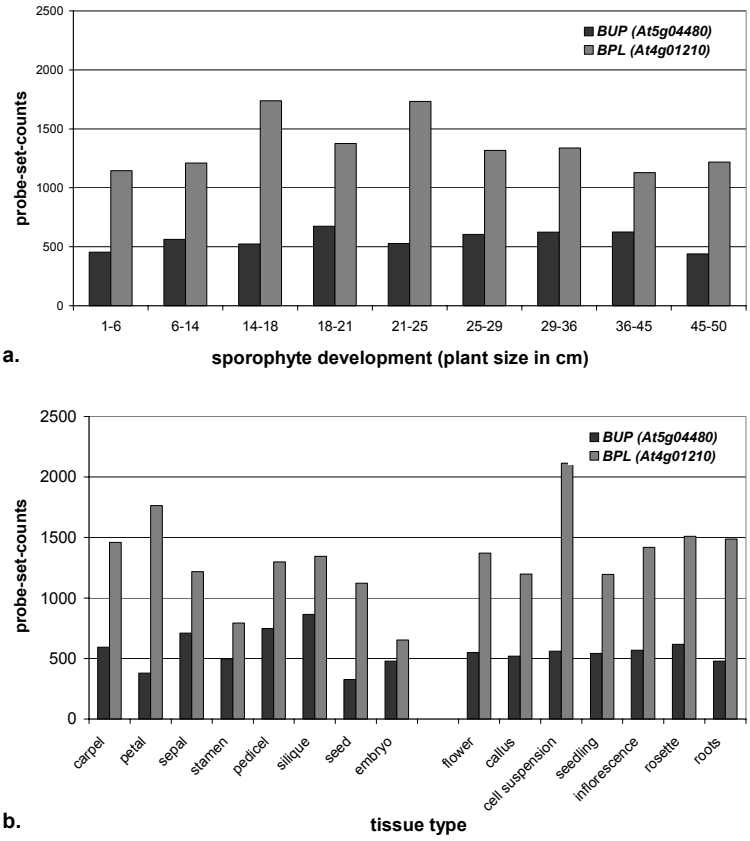

Figure 4: Genevestigator gene expression analysis

a. Developmental and b. tissue specific expression pattern analysis of *BUP* and *BPL*. Data obtained from Genevestigator database (Zimmermann *et al.*, 2004), using all 1434 ATH1 array slides. Data normalized to target value (TGT)=1000

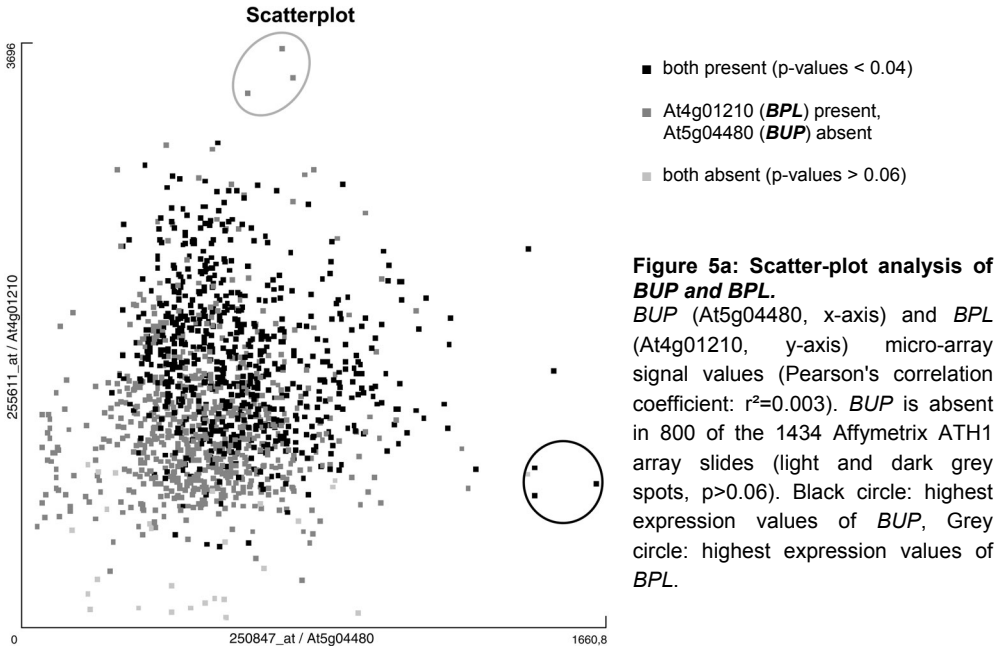

Figure 5a: Scatter-plot analysis of *BUP and BPL.*
BUP (At5g04480, x-axis) and *BPL* (At4g01210, y-axis) micro-array signal values (Pearson's correlation coefficient: r^2=0.003). *BUP* is absent in 800 of the 1434 Affymetrix ATH1 array slides (light and dark grey spots, p>0.06). Black circle: highest expression values of *BUP*, Grey circle: highest expression values of *BPL*.

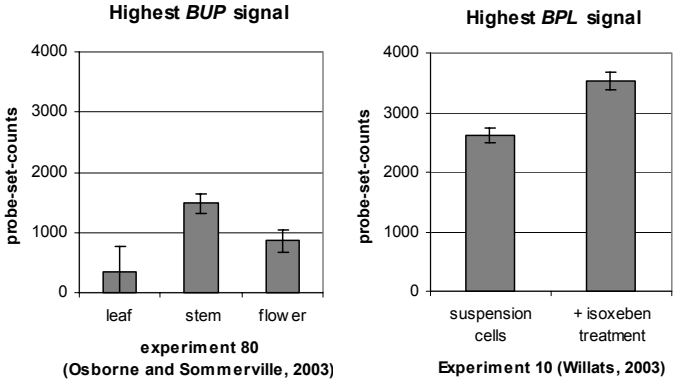

Figure 5b: Highest expression levels of *BUP* and *BPL*
Experiments showing the highest expression values of respectively *BUP* and *BPL*. Data obtained from Genevestigator database (Zimmermann *et al.*, 2004), Expression values ± standard deviation. Data normalized to target value (TGT)=1000

Table 2: Micro-array data on *BUP* and *BPL* expression in pollen

Gene Name	pollen developmental stages				COT	LEF	PET	STM	ROT	RHR	SUS
	UNM	BCP	TCP	MPG							
BUP	0	0	0	0	0	152.0	152.2	135.1	131.2	0	0
BPL	269.9	228.9	0	0	274.3	236.0	317.2	221.0	304.9	201.2	446.4

BUP (At5g04480) and *BPL* (At4g01210) expression in uninucleate microspore (UNM), bicellular pollen (BCP), tricellular pollen (TCP) and mature pollen (MPG), and in the sporophytic samples cotelydons (COT), leaves (LEF), petioles (PET), stem (STM), roots (ROT), root hairs (RHR) and suspension cells (SUS). Data from Honys and Twell (2004).

Gametophytic-sporophytic expression of the GT1 family

To date, the glycosyl transferase family 1 consists of 109 annotated *Arabidopsis* genes (*http://www.Arabidopsis.org/info/genefamily/Glycosyltransferase.html*), of which 101 are represented on the Affymetrix ATH1 array (table 3). 24 GT1 genes were shown to be expressed during pollen development (Honys and Twell, 2004). However, only one of these (At5g54010) was expressed specifically in pollen, during the first two stages of pollen development. The other pollen expressed GT1 genes were also expressed in one or more of the sporophytic tissues (table 3). The GT1 gametophytic/sporophytic gene expression found with the ATH1 array was consistent with the data from the AG array analysis that contained nine annotated GT1 members, of which two were shown to be expressed in pollen (table 3, section b, Honys and Twell, 2003, 2004).

Table 3: Glycosyl transferase family 1 gene expression in pollen and sporophytic issues

genes	Cluster	UNM	BCP	TCP	MPG	COT	LEF	PET	STM	ROT	RHR	SUS
***BUP* (At5g04480)**	**-**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**152,0**	**152,2**	**135,1**	**131,2**	**0,0**	**0,0**
***BPL* (At4g01210)**	**29**	**269,9**	**228,9**	**0,0**	**0,0**	**274,3**	**236,0**	**317,2**	**221,0**	**304,9**	**201,2**	**446,4**
GT1 annotated genes	**Cluster**	**UNM**	**BCP**	**TCP**	**MPG**	**COT**	**LEF**	**PET**	**STM**	**ROT**	**RHR**	**SUS**
At5g54010 [a]	**29**	**399,6**	**416,2**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**
At2g28080 [b]	**2**	**308,5**	**281,5**	**2031,5**	**1957,0**	**382,0**	**172,7**	**270,7**	**284,7**	**340,2**	**428,3**	**863,3**
At4g01070	**2**	**0,0**	**0,0**	**465,4**	**281,3**	**139,1**	**1147,7**	**614,4**	**1541,9**	**1101,3**	**940,8**	**2352,6**
At1g24100	**-**	**0,0**	**0,0**	**0,0**	**0,0**	**371,0**	**1271,2**	**1033,6**	**858,7**	**1385,5**	**948,7**	**232,4**
At2g31790	**-**	**0,0**	**0,0**	**0,0**	**0,0**	**277,3**	**1340,2**	**1265,2**	**855,2**	**494,0**	**416,2**	**0,0**
At2g30150	**-**	**0,0**	**0,0**	**0,0**	**0,0**	**305,1**	**203,3**	**210,0**	**96,9**	**104,8**	**56,2**	**176,4**
At2g29730	**-**	**0,0**	**0,0**	**0,0**	**0,0**	**210,9**	**153,0**	**230,1**	**483,0**	**176,9**	**556,9**	**0,0**
At4g15490	**-**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**737,5**	**228,5**	**368,3**	**238,5**	**0,0**	**37,9**
At3g55700	1	0,0	0,0	57,4	184,6	0,0	28,9	0,0	0,0	35,2	45,1	195,9
At5g66690	3	0,0	65,4	94,2	145,2	148,6	70,6	59,2	66,6	812,5	2389,2	82,5
At3g16520	3	124,5	113,3	116,1	211,5	1168,9	2268,5	2285,3	1083,0	239,5	218,4	156,6
At3g07020	6	351,5	365,5	259,6	423,4	0,0	294,9	293,9	393,6	375,3	391,4	0,0
At1g05560	12	0,0	21,9	0,0	34,8	79,0	119,7	34,0	59,8	397,8	0,0	150,4
At3g21560	16	32,7	0,0	0,0	41,4	430,0	658,7	187,9	53,8	333,4	1240,1	67,1
At1g22380	17	0,0	0,0	0,0	120,9	0,0	84,5	0,0	0,0	72,6	0,0	0,0
At2g15480	17	0,0	0,0	0,0	55,4	0,0	0,0	0,0	0,0	448,0	146,5	140,8
At1g78280	22	0,0	400,7	555,7	0,0	0,0	224,2	231,7	324,1	488,5	398,9	628,5
At1g43620	25	294,8	266,6	99,3	104,6	146,7	248,3	332,7	151,7	140,8	134,0	98,5
At2g26480	27	131,1	142,2	107,8	0,0	0,0	124,1	109,6	0,0	287,7	241,7	73,4
At1g07250	27	202,6	281,5	238,6	0,0	372,2	714,2	536,7	363,7	495,7	416,5	238,5
At1g05570	27	314,3	306,0	196,9	0,0	537,2	652,2	762,7	1504,2	675,5	1368,9	1279,0
At5g59520	29	165,5	165,0	0,0	0,0	485,4	187,5	111,2	162,0	2232,0	688,1	770,6
At4g14100	29	373,3	356,8	0,0	0,0	477,2	333,2	347,5	139,7	0,0	0,0	0,0
At2g31750	34	105,7	0,0	104,7	0,0	1002,6	540,9	247,7	100,9	726,1	429,9	97,8
At3g46720	37	64,6	0,0	0,0	0,0	0,0	0,0	0,0	0,0	131,9	167,9	0,0
At1g64910	39	0,0	36,2	0,0	0,0	23,5	33,1	0,0	35,8	45,1	70,5	0,0
At3g21760	39	0,0	57,6	0,0	0,0	411,6	539,0	349,5	310,7	0,0	0,0	0,0
At4g15260	39	0,0	42,3	0,0	0,0	101,3	112,1	88,1	0,0	308,4	0,0	101,9
At1g01390	-	0,0	0,0	0,0	0,0	182,7	177,7	0,0	0,0	0,0	0,0	0,0
At1g01420	-	0,0	0,0	0,0	0,0	0,0	123,2	158,2	0,0	123,1	98,1	0,0
At1g05530	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	72,6	43,5	0,0
At1g06000	-	0,0	0,0	0,0	0,0	466,5	0,0	132,9	0,0	808,6	1756,4	0,0
At1g07240	-	0,0	0,0	0,0	0,0	112,7	181,9	244,2	51,3	292,8	214,7	0,0
At1g07260	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	223,0	428,6	0,0
At1g10400	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At1g22340	-	0,0	0,0	0,0	0,0	0,0	119,2	0,0	0,0	0,0	0,0	0,0
At1g22360	-	0,0	0,0	0,0	0,0	140,6	176,7	274,9	0,0	369,1	227,5	235,4
At1g22400	-	0,0	0,0	0,0	0,0	293,7	249,9	0,0	0,0	211,9	0,0	127,2
At1g30530	-	0,0	0,0	0,0	0,0	196,4	139,7	267,6	151,0	0,0	0,0	0,0
At1g50580	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	77,5	104,7	0,0
At1g51210	-	0,0	0,0	0,0	0,0	232,0	262,6	0,0	232,3	225,8	242,0	130,6
At1g64920	-	0,0	0,0	0,0	0,0	69,1	0,0	0,0	38,9	106,2	85,9	0,0
At1g73880	-	0,0	0,0	0,0	0,0	1298,9	1322,5	0,0	0,0	57,1	53,5	0,0
At2g15490	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	675,7	0,0	760,1
At2g16890	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	262,0	0,0	0,0
At2g18570	-	0,0	0,0	0,0	0,0	0,0	166,3	213,5	406,1	213,3	0,0	0,0

table 3 continued

GT1 annotated genes	Cluster	UNM	BCP	TCP	MPG	COT	LEF	PET	STM	ROT	RHR	SUS
At2g22590	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	85,3	0,0	0,0
At2g22930	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	109,6	246,6	0,0
At2g23210	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At2g23250	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At2g23260	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At2g29740	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	80,5	492,3	0,0
At2g29750	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	464,6	2089,1	0,0
At2g30140	-	0,0	0,0	0,0	0,0	517,3	1011,2	264,2	281,6	750,5	0,0	1053,9
At2g36750	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At2g36760	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At2g36790	-	0,0	0,0	0,0	0,0	61,0	0,0	0,0	0,0	0,0	0,0	0,0
At2g36970	-	0,0	0,0	0,0	0,0	112,2	304,1	188,2	247,2	102,0	0,0	106,1
At2g43820	-	0,0	0,0	0,0	0,0	169,5	384,3	0,0	0,0	585,7	303,7	574,9
At2g43840	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	128,1	130,3	0,0
At3g02100	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	71,2	0,0	0,0	0,0
At3g21750	-	0,0	0,0	0,0	0,0	336,3	406,1	257,9	282,9	119,3	97,3	0,0
At3g21770	-	0,0	0,0	0,0	0,0	433,3	0,0	95,0	194,6	1213,2	2271,1	335,0
At3g21780	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At3g22250	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	144,0	118,1	89,4
At3g46650	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	69,9	0,0	0,0
At3g46660	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	71,4	0,0	0,0
At3g46670	-	0,0	0,0	0,0	0,0	162,5	211,1	130,8	0,0	0,0	0,0	0,0
At3g46680	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At3g46690	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At3g46700	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	633,8	257,8	0,0
At3g50740	-	0,0	0,0	0,0	0,0	0,0	643,3	449,1	0,0	777,6	104,4	0,0
At3g53160	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	102,8	0,0	0,0
At3g55710	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At4g09500	-	0,0	0,0	0,0	0,0	0,0	0,0	219,1	43,6	0,0	0,0	0,0
At4g15270	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	85,6	78,2	116,2
At4g15280	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At4g15480	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	183,0	0,0
At4g15500	-	0,0	0,0	0,0	0,0	0,0	0,0	145,3	66,2	379,5	671,3	0,0
At4g15550	-	0,0	0,0	0,0	0,0	290,3	510,8	0,0	0,0	536,8	131,2	71,1
At4g27570	-	0,0	0,0	0,0	0,0	37,0	0,0	439,2	60,8	333,5	186,0	0,0
At4g36770	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At5g03490	-	0,0	0,0	0,0	0,0	0,0	200,7	0,0	149,2	141,7	0,0	0,0
At5g05860	-	0,0	0,0	0,0	0,0	386,6	0,0	0,0	92,9	225,7	216,8	0,0
At5g05870	-	0,0	0,0	0,0	0,0	237,0	172,7	192,6	172,9	98,0	50,8	0,0
At5g05880	-	0,0	0,0	0,0	0,0	296,2	0,0	0,0	0,0	247,1	444,9	0,0
At5g05890	-	0,0	0,0	0,0	0,0	603,8	203,9	0,0	0,0	284,5	171,3	0,0
At5g05900	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At5g12890	-	0,0	0,0	0,0	0,0	243,8	307,5	315,0	447,9	177,8	0,0	577,2
At5g14860	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At5g17030	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At5g17040	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At5g17050	-	0,0	0,0	0,0	0,0	464,7	526,5	152,9	367,4	863,2	1247,6	0,0
At5g26310	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	172,8	267,1	0,0
At5g38035	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At5g49690	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	282,7
At5g53990	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	143,2	130,1	0,0
At5g54060	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
At5g59530	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	452,2	0,0	0,0
At5g65550	-	0,0	0,0	0,0	0,0	0,0	60,0	61,5	0,0	0,0	0,0	58,7
At1g22370	-	0,0	0,0	0,0	0,0	0,0	238,0	0,0	0,0	0,0	0,0	0,0

[a] pollen-specific glycosyl transferase 1 (GT1), [b] GT1s also represented on the AG array. Clusters 1,2,3,6,12,16,17,22,25,27,29,34,37and 39: distinct expression patterns during pollen development (see Honys and Twell, 2004). Data from Honys and Twell (2004). Samples used are uninucleate microspore (UNM), bicellular pollen (BCP), tricellular pollen (TCP) and mature pollen (MPG), and the sporophytic samples for gametophyte-sporophyte expression comparison are cotelydons (COT), leaves (LEF), petioles (PET), stem (STM), roots (ROT), root hairs (RHR) and suspension cells (SUS).

Discussion

Promoter fusion analysis

To investigate the expression of *BURSTING POLLEN (BUP)*, a gene important during pollen germination and pollen tube growth, promoter fusion constructs were made, and northern blot and RT-PCR analyses were performed.

In planta gene expression analysis using the promoter-GFP fusion of the pCAMBIA 1302 plasmid resulted in no GFP signal in *BUP::GFP* transformants, while pollen tubes with the *LAT52::GFP* fusion showed a clear GFP signal. This indicated that the *BUP* promoter is too weak to give a visible GFP signal, above the background level of cell wall autofluorescence. This problem using GFP fused to a (weak) promoter was also discussed previously by Mantis *et al.* (2000), and they concluded that the use of GUS as marker protein was advantageous and promoter activity was easier to detect using GUS. The pCAMBIA 1303 bifunctional reporter construct was used for creating promoter-*GUS:GFP* fusion plants. Unfortunately, the expression analysis of three different constructs, using the *35S* promoter, the *LAT52* promoter and the *BUP* promoter respectively, gave similar GUS staining patterns, which may result from an artefact. We believe that the presence of a very strong constitutive promoter (like the *35S* promoter) on the T-DNA nearby the promoter-reporter gene fusion interferes with the expression of this reporter gene. This was not the case when a weaker constitutive promoter (like the *NOS* promoter) was used to drive the plant selection marker (Twell *et al.,* 1990). The pCAMBIA 1302 and 1303 vectors used here, with the *35S* promoter driving the Hygromycin resistance gene, turned out not to be suitable for analysis of promoter activity, which was also recently acknowledged on the CAMBIA website (*http://www.cambia.org/main/r_et_camvec. htm*). However, we can still conclude from these experiments that the *BUP* promoter is a relatively weak promoter: No signal was visible in pollen, while the *LAT52* promoter fusion plants did show GUS staining in pollen in addition to the *35S* promoter derived ectopic GUS expression.

BUP and *BPL in vivo* and *in silico* expression analysis

Northern blot and RT-PCR analyses have shown that *BUP* is constitutively expressed in pollen and in sporophytic tissues. *BUP* expression in pollen, though at a very low level, agrees with the result that *BUP* is essential for pollen germination and pollen tube growth (Chapter 4) and that a mutation in this gene severely affects male gametophyte fitness (Chapter 3). Also micro-array based data on *BUP* expression in the Genevestigator database consistently showed low but constitutive expression of the *BUP* gene in sporophytic tissues. In the pollen transcriptome micro-array experiment by Honys and Twell (2004), however, no expression of *BUP* was found at any of the four successive pollen developmental stages. This inconsistency with the RT-PCR result is most likely to be explained by

the higher sensitivity for detecting a very low abundant transcript with the PCR-based assay.

The *BUP* gene product has one *Arabidopsis* homologue, encoded by At4g01210 (*BPL*), which is also a family 1 glycosyl transferase (35% similarity, Chapter 3). This suggested that *BUP* and *BPL* might have a similar similar function, though they are not likely to be fully redundant. Unfortunately, no *bpl* mutants were available to test whether it also showed segregation distortion (Chapter 3). RT-PCR analysis of *BPL* showed no expression in mature pollen, but the gene was expressed in the sporophytic tissues. Micro-array data from Genevestigator confirmed this constitutive expression in the sporophyte, which was at a higher level than *BUP*. The micro-array data of *BPL* pollen expression (Honys and Twell, 2004) also showed that it is an early pollen gene, expressed in microspores but repressed after pollen mitosis. As the RT-PCR analysis was on mature pollen, the absence of a *BPL* signal is consistent with these micro-array data. Apparently, *BUP* and *BPL* are both expressed constitutively in the sporophyte, but they show a differential expression pattern during pollen development, indicating a different role in pollen development and function.

Though *BUP* and *BPL* are not among the 121 *Arabidopsis* glycosyl transferase 1 (GT1) proteins annotated in the Cazy database (Cazy database, http://afmb.cnrs-mrs.fr/CAZY/), protein structural analysis have shown that these genes belong to the glycosyl transferase 1 family (Egelund *et al.*, 2004). Compared to the 60-90% overlap of overall gametophyte and sporophyte gene expression (Honys and Twell, 2003, 2004), the GT1 family, with only 23% overlap, has relatively few genes expressed in pollen. However, it is possible that some of the 'sporophyte-specific' GT1 genes are also expressed in pollen but are simply not detected because their low abundance, similar to *BUP*.

The putative function of *BUP* and *BPL*

Glycosyl transferases are involved in the synthesis of glycoproteins, glycolipids, polysaccharides and cell wall components. The highest expression level of *BUP* in the Genevestigator database was in stem tissue. The stem is a tissue type in which the rigidity of the cell wall is obviously an important factor. Stem cells, perhaps more than other cells, need to have a strong but flexible cell wall, and genes necessary for their synthesis might therefore be upregulated in this tissue. *bup* mutant pollen show a defect in pollen germination and pollen tube initiation, which could be caused by a defect in pectin structure of the pollen and pollen tube wall (Chapter 4). Like cells of the stem, pollen grains sustain a high turgour pressure during pollen hydration and germination, which coincides with changes in the pectin molecular structure (Van Aelst and Van Went, 1992). In fact, pollen tubes of the *Arabidopsis* mutant *vanguard,* mutated in a gene coding for a pectin methylesterase, show a 'burst' phenotype similar to that of *bup* pollen when grown

in vitro, supporting that pectin is important for the strength of the pollen cell wall (Jiang *et al.,* 2005).

The highest expression level of *BPL* was found in micro-array experiments aimed at investigating gene regulation of cell wall synthesis (Manfield *et al.*, 2004). *BPL* expression was significantly increased when *Arabidopsis* suspension cells were treated with the herbicide isoxaben, a strong inhibitor of the cellulose synthesis. Suspension cells habituated to isoxaben can compensate for the disruption of cellulose synthesis by the synthesis of other cell wall components like pectin (Manfield *et al.*, 2004). All together, this indicates a role of the glycosyl transferases BUP and BPL in the glycosylation of pectin or other cell wall components.

Pollen selection and gametophytic/sporophytic expression

From an evolutionary perspective, competition between pollen on a single pistil to fertilize the limited amount of embryo sacs will lead to a strong selection of pollen-expressed genes that increase pollen fitness (Mulcahy *et al.*, 1996). The 'late' pollen genes that are specifically and very highly expressed in pollen are thought to encode proteins that affect this fitness, like genes involved in signalling and in cell wall synthesis (Honys and Twell, 2003; Becker *et al.*, 2003; Lee *et al.*, 2003). On the other hand, the significant overlap of pollen-expressed and sporophyte-expressed genes (60-90%) may provide for the selection of sporophytic expressed genes in the haploid pollen during the reproductive processes (Honys and Twell, 2003; Becker *et al.*, 2003). T-DNA insertional mutation of the pollen-expressed *BUP* gene resulted in a severe reduction in fitness. As this gene is not redundant in pollen and is expressed in vegetative tissues, the strong selection against a mutation of this gene during sexual reproduction might suggest that *BUP* is essential for the sporophyte as well, even though the female fitness of the mutant was not affected. No recessive mutant phenotype in homozygous mutants could be analysed to support this hypothesis, indicating that the homozygous mutant might indeed be embryo lethal. To support this, the T-DNA insertional mutant of another glycosyl transferase, AtCSLA7 (a cellulose synthase-like putative glycosyl transferase involved in the synthesis of cell wall polysaccharides), too was only affected in the male T-DNA transmission and not the female transmission. For this glycosyl transferase, embryo lethality of the homozygous insertion was documented in detail (Goubet *et al.*, 2003). It is noteworthy that pollen grains undergo a dramatic dehydration and rehydration during development and germination, and may need a well-built cell wall structure to sustain this. In contrast, the female gametophyte is embedded and supported by the surrounding sporophytic tissue and therefore may be less sensitive to mutations in genes involved in the cell wall synthesis.

We have shown that the genes coding for the homologous GT1 glycosyl transferases BUP and BPL both are expressed during pollen development and in the sporophyte. *BUP* is essential for pollen germination and is likely to have an important role in the sporophyte metabolism as well. *BPL* is expressed earlier in pollen development, but as we do not have a mutant for this gene we cannot say whether it plays an important role in pollen function. Several results support the hypothesis that both *BUP* and *BPL* may be involved in the synthesis of cell wall components, in particular the synthesis of pectin.

Chapter 6

Protein localization analysis of BURSTING POLLEN (BUP), a putative glycosyl transferase involved in pollen germination and pollen tube growth

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Abstract

BURSTING POLLEN (BUP) is shown to be essential for pollen germination and pollen tube growth. BUP resembles a family 1 glycosyl transferase protein, as it has a short amino-terminal cytoplasmic tail, a single transmembrane domain and a large luminal cytoplasmic domain containing a domain that is highly homologous to the glycosyl transferase family 1 domain. Glycosyl transferases that have this typical type II transmembrane protein structure are mostly located in the Golgi bodies, which would be consistent with a putative role for BUP in the synthesis of carbohydrous cell wall components like pectin and hemicellulose. Experimental analysis of the subcellular localization of BUP was performed using amino-terminal GFP fusion proteins, and by western blot and immuno localization analyses using BUP and GFP antibodies. Co-localization of the BUP-GFP fusion protein with the Golgi marker ST-RFP in onion epidermal cells showed that BUP-GFP was indeed labelling Golgi bodies, though strand-like structures that appear to be part of the cortical cytoskeleton too were labelled with GFP. Consistently, western blot analysis of transformed onion and *Arabidopsis* cells using the GFP antibody showed that the BUP-GFP fusion protein was targeted to membranes, while some of the fusion protein was modified to produce a soluble form of GFP suggesting proteolytic cleavage. Unfortunately, due to the insufficient quality of the peptide antibodies raised against BUP, immuno localization and western blot analysis using these antibodies could not confirm Golgi localization of the native BUP protein in pollen tubes or leaves of *Arabidopsis*, nor could it give any more information on the occurrence of proteolytic cleavage of this protein *in vivo*.

Introduction

Glycosyl transferases are enzymes that attach a sugar molecule to a protein, carbohydrate or lipid, thereby creating a glycosidic bond. In plants, glycosyl transferases are involved in the synthesis of polysaccharides like the cell wall components and in the posttranslational modification of proteins. For example, cellulose synthases and callose synthases are glycosyl transferases with multiple membrane-spanning domains, localized in the plasma membrane, that synthesize the cellulosic and callosic cell wall components (Delmer, 1987; Kudlicka and Brown, 1997; Richmond, 2000; Verma, 2001). Most other glycosyl transferases, however, are single transmembrane proteins of the type II orientation. In contrast to type I single membrane-spanning proteins the amino-terminus of type II transmembrane proteins is located on the cytoplasmic side of the membrane (see also figure 4a of chapter 1 on page 18). Type II glycosyl transferases are generally localized in the secretory pathway, in the endoplasmatic reticulum (ER) and in particular the Golgi apparatus, in the same sequence in which they act during protein glycosylation and glycan synthesis. Thus, enzymes that act early in glycan biosynthesis are localized to cis and medial compartments, whereas enzymes that act later in the biosynthetic pathway tend to localize in the trans-Golgi cisternae (reviewed in Fukuda, 1994).

Much effort was made during the 1990s to characterize targeting signals that explain the subcellular and sequential localization of these glycosyl transferases within the different Golgi compartments (reviewed in Young, 2004). Like the signal peptides for ER localized proteins (Munro and Pelham, 1987; Gomord *et al.,* 1999; Pagny *et al.,* 1999) one consensus sequence near the transmembrane domain (S/T-X-E/Q-R/K) was proposed to act as a Golgi-retention signal in some Golgi-associated glycosyl transferases (Bendiak *et al.,* 1990). Also in other Golgi-resident enzymes this sequence has been found (Slentz-Kesler *et al.,* 1998). However, though glycosyl transferases with a similar function are highly conserved between species, different glycosyl transferases of the same protein family do not show a high level of sequence similarity. Most localization studies of Golgi-resident glycosyl transferases therefore conclude that Golgi localization can also be regulated by the domain structure and by posttranslational modifications of glycosyl transferases (reviewed in Young, 2004).

Golgi-localized glycosyl transferases are characterized by a short amino-terminal cytoplasmic tail, a hydrophobic transmembrane domain, a large carboxy-terminal catalytic domain localized in the Golgi lumen, and a luminal stem region that connects the catalytic domain to the transmembrane domain. The domains required for Golgi retention have been extensively studied in several glycosyl transferases. To do this, mutant and chimeric fluorescent proteins were created and localized using immuno-fluorescence microscopy or immuno-electron

microscopy. These studies have demonstrated that the Golgi apparatus retention signal of these glycosyl transferases must reside in the amino-terminal part of these enzymes, which includes the amino-terminal cytoplasmic tail, the transmembrane domain and the stem region (reviewed in Paulson and Colley, 1989).Though most of these experiments were performed with mammalian glycosyl transferases, the Golgi-localization signal appears to be similar in plants, as the transmembrane domain region of the rat α-2,6-sialyltransferase (ST) is also targeted to subcompartments of the plant Golgi apparatus (Wee *et al.,* 1998), and studies with plant glycosyl transferases also gave similar results (Essl *et al.,* 1999, Strasser *et al.,* 2000; Milland *et al.,* 2001; Dirnberger *et al.,* 2002; Pagny *et al.,* 2003). The importance of the cytoplasmic tail, transmembrane domain and stem region for glycosyl transferase localization is consistent with the two existing models for Golgi-retention: (1) Retention through oligomerization and (2) Retention through differences in the membrane thickness along the exocytic pathway. The first model involves the formation of homo- or hetero-oligomers within Golgi cisternae, by the formation of cysteine bonds between two glycosyl transferase proteins. These oligomers would be too large to be transported through vesicles and therefore would remain in the Golgi compartments (Nillson *et al.,* 1994; Machamer, 2001). The cysteine bonds can be formed in different domains of the glycosyl transferase, and are also found in the stem region. The involvement of the stem region in dimerization might explain the importance of this region for the localization of some glycosyl transferases (Nilsson *et al.,* 1993, 1994; Weisz *et al.,* 1993). The second, membrane thickness model (Bretscher and Munro, 1993) on the other hand, is consistent with the importance of the transmembrane domain for localization. This model proposes that the relatively short transmembrane domain of Golgi proteins prevents them from entering the cholesterol-rich transport vesicles that would transport them to the plasma membrane. This has been shown to be true for type I transmembrane proteins, with a transmembrane domain of 17 amino acids for ER, 20 amino acids for Golgi and 23 amino acids for the plasma membrane (Brandizzi *et al.,* 2002), as well as for type II transmembrane proteins such as the syntaxins (Watson *et al.,* 2001).

Within the cell, the Golgi apparatus is organized in a polarized stack of the cis, median and trans cisternae (reviewed in Hawes, 2005). Studies using Golgi targeted fluorescent protein constructs have shown that these stacks are associated with tubules of the cortical ER network (Boevink *et al.,* 1998). In addition, the Golgi bodies were shown to be moving along actin filaments, which are overlaid by the ER network (reviewed in Hawes, 2005).

Here we analyse the subcellular localization of the *Arabidopsis* protein BURSTING POLLEN (BUP), a protein involved in pollen germination and pollen tube growth. The strong resemblance of this type II membrane protein with the structure of known Golgi-resident glycosyl transferases, suggests that BUP is a

functional glycosyl transferase family member. Homology with domains like the glycosyl transferase group 1 domain and the activated nucleotide binding site, as well as indications that BUP might be involved in cell wall synthesis strongly support this hypothesis. To analyse whether BUP is indeed targeted to the Golgi, protein-GFP fusion constructs were made, peptide antibodies were raised against the BUP protein and western blot and immuno localization analyses were performed to establish its subcellular localization.

Material and methods

Plant Growth and Transformation

The *Arabidopsis thaliana* (ecotype Columbia) plants used in this study were sown on a 3:1 compost:vermiculite mixture and, after three days vernalization at 4°C in the dark, grown under greenhouse conditions with supplemental lighting (16 h of light at 22°C). Transgenic *Arabidopsis* lines were generated by *Agrobacterium tumefaciens* mediated transformation using the floral dipping technique as described by Clough and Bent (1998) and in Chapter 3. T1 seeds were grown on agar plates containing 0.5x MS salt including B5 vitamins (pH 6.0), MES buffer, 0.8% (w/v) select agar and 20 mg/l Hygromycin and resistant seedlings were transplanted to soil. *35S::BUP:GFP* (G) and *35S::ST:RFP* (R) plants were crossed to create transformants carrying both constructs. As both T-DNAs confer Hygromycin resistance, only 1/3 of the resistant F1 seedlings carried both T-DNA's. When selfed, 9/16 of the progeny would carry both constructs. The expected frequency of of the plants carrying both constructs in the total F2 population is 1/3x9/16= 3/16, and the expected number in progeny of 33 Hygromycin resistant F1 plants therefore is 3/16x33=6.

Construction of 35S::BUP:GFP and LAT52::BUP:GFP fusion protein constructs

Molecular cloning techniques were performed as described by Sambrook *et al.* (1989). The 515 bp *BUP*-gene fragment encoding the N-terminal BUP fragment was amplified from the complementation construct '+04480' (Chapter 3) using the primers 5'-ATCACTAATAGTGACAATTACTGCT-CAAGAAAGC-3' and 5'-TGAA**ACTAGT**CACGAGAGCTAGCCTGGGCGGACG-3' (with SpeI restriction site added in bold). For the *35S::BUP:GFP* construct, the PCR-fragment was digested with NcoI and SpeI and ligated into the NcoI and SpeI sites of pCAMBIA1302. For the *LAT52::BUP:GFP* construct, the plasmid pLAT52.7 (D.Twell) and the *35S::BUP:GFP* construct were digested with SalI and NcoI, and the SalI-NcoI LAT52 promoter fragment was ligated into the BUP:GFP 1302 plasmid, replacing the 35S promoter. The constructs were transferred into *Agrobacterium tumefaciens* strain GV3101 by freeze thaw transformation (Chen *et al.,* 1994).

Biolistic Transformation

The plasmids with the *35S::GONST:YFP* and *35S::ST:RFP* constructs were generously provided by Dr. P. Dupree (Cambridge, UK) and C. Saint-Jore (Oxford, UK), respectively. Plasmid DNA (5 µg) was delivered into onion (*Allium cepa*) epidermal cells using tungsten particle bombardment. For experiments in which BUP:GFP and ST:RFP were expressed simultaneously, 2.5 µg of each plasmid was bound to the tungsten microprojectiles. Onion bulbs were purchased from a local vegetable store, and inner epidermal peels were placed on agar plates containing 4.4 g/l MS salts, 30 g/L sucrose, and 7% select agar, pH 5.7. Peels were bombarded within 1 hour of transfer to agar plates. Tungsten microprojectiles (1.1 µm; Bio-Rad) were coated with DNA according to the manufacturer's instructions. Microprojectiles were bombarded into the onion epidermal cells using a Biolistic PDS-1000/He system (Bio-Rad) with 900-p.s.i. rupture discs under a vacuum of 28 in Hg. After bombardment, the cells were allowed to recover for 24-48 hr on agar plates at 22°C under a 16h photoperiod regime before being viewed under a fluorescence microscope and confocal laser scanning microscope.

Peptide-antibody synthesis

For peptide-antibody synthesis, an amino-terminal (AcNH-**VRNSLSLEIDDNGGA**C-COOH) and carboxy-terminal peptide (H2N-C**YFRIKNGYKQKSLGG**-COOH) were synthesized and coupled to the carrier Keyhole Limpet Hemocyanin (KLH) mediated by m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) (Eurogentec, Belgium). The BUP immune sera were obtained from Davids Biotechnologie (Regensburg, Germany) and produced by immunization of chicken (pre-selected for absence of background pre-immune signals on blots and immuno-detection slides) with the two individual peptide-

KLH conjugates. These N-BUP (amino-terminal peptide) and C-BUP (carboxy-terminal peptide) immune sera were purified according to the standard procedures. The N-BUP antiserum was later affinity-purified against the peptide without KLH (VRNSLSLEIDDNGGA) to remove aspecific antibodies, resulting in the purified N*-BUP antibody fraction.

Protein isolation and western blot analysis

Proteins of onion epidermal cells and *Arabidopsis* leaves were extracted in a homogenization buffer (330 mM sucrose, 100 mM KCl, 1mM EDTA, 50 mM Tris, 0,05% MES, 5mM DTT, Complete ® proteinase inhibitor cocktail (Roche Mannheim), pH 7.5). The homogenate was centrifuged at 1000g for 15 minutes to collect intact cells and debris, and the supernatant was centrifuges at 10000g for 15 minutes to collect the large cellular organelles (organelle fraction). Finally, the microsomal fraction was pelleted by centrifugation of the supernatant at 20000g for 75 minutes. The microsomal fraction and the organelle fraction were dissolved in membrane buffer (330 mM sucrose, 200 mM DTT, 25 mM Tris, pH 8.5). The soluble proteins in the supernatant (the cytoplasmic fraction) were precipitated with acetone and also dissolved in membrane buffer.

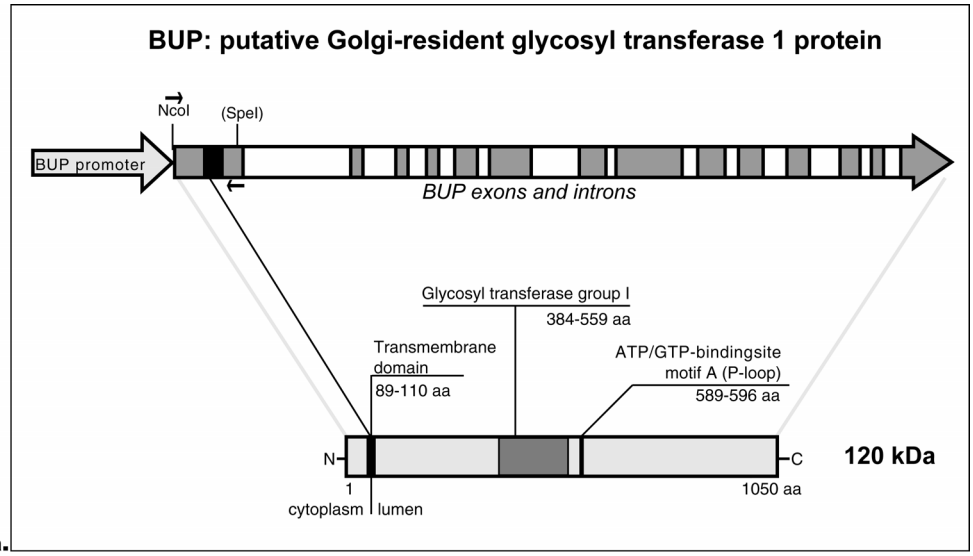
5-20 µg of protein was separated by 10-12% SDS-PAGE. The proteins were electroblotted onto a nitrocellulose membrane in 39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol, pH 8.3. For the peptide spots, 1 µg of the peptide-conjugates or KLH was spotted onto nitrocellulose membrane, and allowed to dry. For detection, the membranes first were incubated in blocking buffer (5% non-fat dried milk in PBT) for 3 hours, after which they were incubated for two hours with either N-BUP serum (1:200 in blocking buffer), C-BUP serum (1:100 in blocking buffer), purified N*-BUP (1:20 in blocking buffer) or anti-GFP (Molecular Probes) (1:500 in blocking buffer). After incubation with the first antibody, the membranes were rinsed and incubated with the secondary antibody, horse-radish-peroxidase conjugated goat anti-chicken antibody (GAC-HRP) or goat anti-rabbit antibody (GAR-HRP) (Pierce Rockford, Illinois) 1:20000 in blocking buffer for one hour. All incubations were performed at room temperature. The membranes were developed using SuperSignal West Pico Chemiluminscent substrate (Pierce) according to the manufacturer's protocol and exposed to Kodak X-omat AR scientific Imaging Films. All autoradiograms were digitized and the contrast and brightness were adjusted with Adobe Photoshop.

Immuno-gold labelling

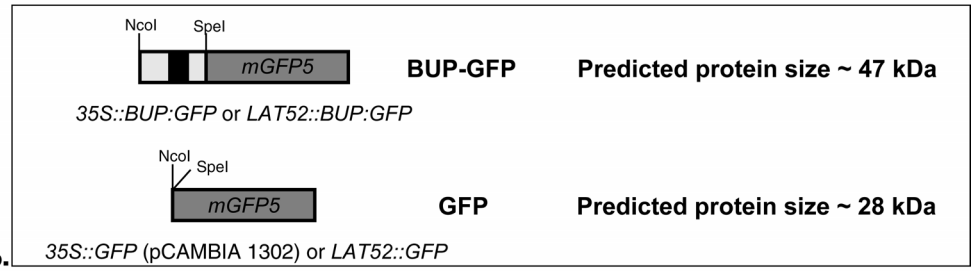
Immuno-localization on sections of leaves, roots and pollen tubes were carried out according to standard procedures. Leaves and roots were fixed in 3% paraformaldehyde and 0.5% glutaraldehyde in 0.05 M phosphate buffer pH 7.2, for 2 hours at room temperature. After 3x rinsing in buffer, the tissue was rinsed in bidest and dehydrated through an ethanol series, at decreasing temperatures (50% ethanol at 4°C, 60% and 75% at -20°C and 90% and 100% at -35°C) and finally embedded in LR White resin. Pollen tubes were grown on pollen germination media as described in Derksen *et al.,* 2002 (Chapter 2). Membranes with pollen tubes grown for about 2-2.5 h were plunged into liquid propane 3.5 (Air Liquide, Belgium; –175°C), transferred into liquid nitrogen and freeze substituted in a Reichert-Jung (Austria) freeze-substitution apparatus. After substitution at –90°C for 36 h in 0.1% uranyl acetate in acetone, the temperature was slowly raised (4°C/h) to room temperature. Membranes with pollen tubes were embedded in LR White resin and the dialysis membrane was removed during the trimming process. Sections were cut on a Sorvall MT-2 ultra-microtome (Sorvall, Norwalk, Conn., USA) using a diamond knife and mounted on formvar-coated nickel grids (Stork-Veco, Eerbeek, The Netherlands).

Ultra-thin sections of leaves and roots were placed in drops of 50 mM glycine in PBS buffer for 15 min at room temperature, after washing in PBS the sections, including the longitudinal sections of pollen tubes, were incubated for 30 minutes in PBS buffer with 5% BSA-c (Aurion) to block non-specific binding. Following 3 washes for 15 minutes in PBS buffer with 0.1% BSA-c the sections were incubated overnight at 4°C with the 1:100 diluted primary antibody N-BUP, C-BUP, N*BUP or 1: 200 diluted antiGFP IgG (Molecular Probes) in PBS buffer with 0.1% BSA-c. The sections were rinsed 3x 15 minutes in buffer with BSA-c and incubated with the second antibody. The sections incubated with the

N-BUP, C-BUP or N*BUP antisera were subsequently incubated for 1 h with 1:400 diluted rabbit anti-chicken IgY (IgG) (H+L) (Jackson Immuno Research) and after washing in buffer, for 1h with 1:400 diluted 10nm gold conjugated protein A IgG. The sections incubated with antiGFP were incubated for 1 h with 1: 400 diluted. All preparations were washed in phosphate buffer for 5 min each, further rinsed in distilled water and air-dried. Subsequently the sections were post-stained with 2% uranyl acetate in H_2O for 15 min and examined in a Jeol transmission electron microscope (100 CX, JEOL, Tokyo, Japan).



a.



b.

BUP-GFP fusion protein sequence

MVRNSLSLEIDDNGGAGRDGNHNANNVAGNGDTSFHSIRDRLRLKRNSSDRRDRSHSGLDRPSLRTRPHHI
GRSLNRKGLLSLLKPRG**TCLLYFLVAFTVCAFVMSSLLL**QNSITWQGNVKGGQVRSQIGLGSTLKYVPG
GIARTLIEGKGLDPLRSAVRIGVRPPRLALV tskgeelftgvvpilveldgdvnghkfsvsgegegdatygkltlkficttgklpvpwptl
vttfsygvqcfsrypdhmkrhdffksampegyvqertiffkddgnyktraevkfegdtlvnrielkgidfkedgnilghkleynynshnvyimadkqk
ngikanfktrhniedggvqladhyqqntpigdgpvllpdnhylstqsalskdpnekrdhmvllefvtaagithgmdelykashhhhhhv

c. Capital letters: BUP N-terminal fragment, **transmembrane domain highlighted**, small grey letters: GFP protein, putative Golgi-retention signal underlined

Figure 1: BUP protein domain structure and protein fusion construct
a. The domain structure of the type II transmembrane protein BUP strongly resembles that of Golgi resident glycosyl transferases. b. BUP-GFP protein construct. The NcoI-SpeI amino-terminal BUP protein coding fragment contained the cytoplasmic tail, the transmembrane domain (black) and part of the luminal stem region. c. Protein sequence of the BUP-GFP protein fusion product, showing the 22 amino acid transmembrane domain in bold.

Results

Subcellular localization of a BUP-GFP fusion protein in onion epidermal cells and *Arabidopsis* root epidermal cells

In order to investigate the subcellular localization of the putative glycosyl transferase BUP (figure 1a), the N-terminal 515 bp fragment of *BUP* was translationally fused to the 5' end of the coding region of the modified green fluorescent protein gene *mGFP5* of pCAMBIA 1302 (Haseloff *et al.,* 1997) (figure 1b). This BUP protein fragment contained the cytoplasmic N-terminal tail, the 22 amino acid predicted transmembrane domain and part of the stem region. BUP does not contain a motif matching the proposed Golgi-retention signal S/T-X-E/Q-R/K near the transmembrane domain (Bendiak *et al.* 1990), though the TWQG sequence at position 115-118 does include two of the three specific amino acids required (figure 1c). The original pCAMBIA 1302 plasmid was used as control for the localization of soluble mGFP. Both GFP constructs were under transcriptional control of the Cauliflower Mosaic Virus 35S promoter (35S), or the pollen-specific LAT52 promoter for analysis of the protein localization in pollen. The correct translational fusion of the BUP and GFP coding sequences was confirmed by restriction enzyme analysis and by nucleotide sequencing. The chimeric BUP-GFP protein was expressed in onion epidermal cells after biolistic transformation as revealed by GFP signal analysis using the fluorescence microscope. This GFP signal pattern was compared with the localization of the Golgi markers GONST-YFP (Baldwin *et al.,* 2001) and ST-RFP (Wee *et al.,* 1998; Saint-Jore *et al.,* 2002) and with that of the soluble mGFP5 protein of pCAMBIA 1302 (figure 2a). Two days after transformation with *35S::GONST:YFP*, the onion cells showed a fine dotted YFP fluorescing pattern. Due to the overlap of the GFP and YFP emission spectra, however, GONST-YFP was not suitable for co-localization of BUP-GFP. The ST-RFP Golgi marker also showed a spotted pattern within the onion cytoplasm, though the presence of higher levels of ST-RFP protein also led to the formation of apparent protein aggregates in some cells. Expression of *35S::BUP:GFP* in onion epidermal cells resulted in the formation of fluorescent GFP dots within the cytoplast, like the two Golgi markers. Co-bombardment with *35S::BUP:GFP* and *35S::ST:RFP* showed co-localization of both fluorochromes, indicating that the BUP-GFP fusion protein is targeted to the Golgi bodies (figure 2b). Analysis at a higher magnification however, showed that BUP-GFP fluorescent Golgi-bodies were in close vicinity to fibre-like strands that were also targeted by the BUP-GFP fusion protein (figure 2c). In the onion epidermal cells, these fluorescing strands appeared to be randomly orientated. As Golgi bodies are known to be associated with the ER, the fluorescing strands could be part of this large endomembrane structure. Alternatively, as the strands are fairly straight, the

GFP could also be labelling components of the cortical cytoskeleton, i.e. the actin filaments or microtubules.

The localization pattern in onion epidermal cells was compared with the localization in chlorophyll-free root epidermal cells of *Arabidopsis* Col-0 plants stably transformed with the *35S::BUP:GFP* construct. Eleven independent *35S::BUP:GFP Arabidopsis* transformants were analysed and though the intensity of the GFP signal between these transformants varied, the pattern was similar in all transformants. The root epidermal cells showed a GFP pattern similar to that in onion cells: GFP labelled dots associated with fluorescing strands. In contrast to the random orientation in onion cells, the strands in root cells appeared to be neatly organized in a radial orientated fibrous network (figure 2d). This strict organization suggested that BUP-GFP was indeed labelling components of the cytoskeleton.

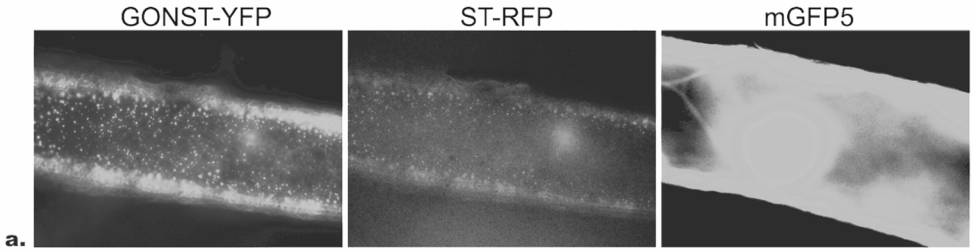
In order to compare the fluorescent pattern of BUP-GFP with that of the ST-RFP Golgi marker in *Arabidopsis*, stable transformants of both fusion proteins were crossed and the progeny of F1 plants were analysed for the presence of both fluorochromes. As both the ST-RFP and BUP-GFP transformants carried the Hygromycin resistance genes for selection, no direct antibiotic selection for the presence of both constructs was possible. Unexpectedly, fluorescence microscopic analysis of the epidermal root cells of progeny of 33 Hygromycin resistant F1 plants, did not show the presence of a transformant carrying both protein-fusion constructs, though the expected number of these plants was six (for the calculation see material and methods). Both *BUP:GFP* and *ST:RFP* were under control of the 35S promoter and one possible explanation of this lack of double transformants might be the occurrence of promoter-silencing.

To analyze the localization of BUP in pollen tubes, *Arabidopsis* plants were stably transformed with the *LAT52::BUP:GFP* construct. Pollen tubes from these plants, grown overnight *in vitro* on germination medium, were analysed by confocal laser scanning microscopy. However, due to the weak GFP signal in pollen tubes and the low laser intensity and resolution of the microscope used, the fluorescent signal was difficult to visualise.

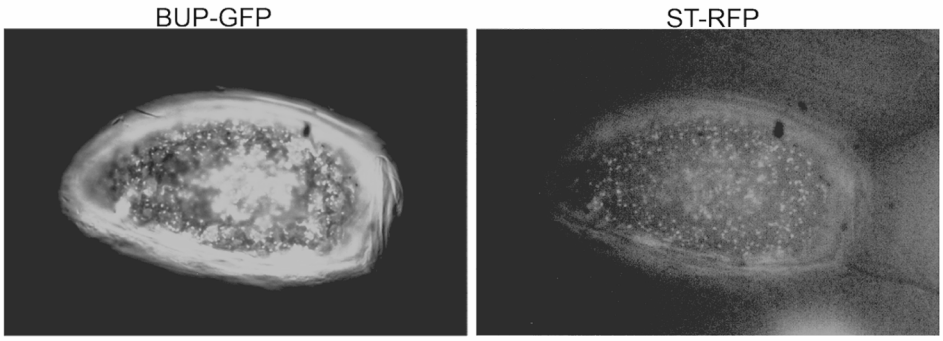
Next page:

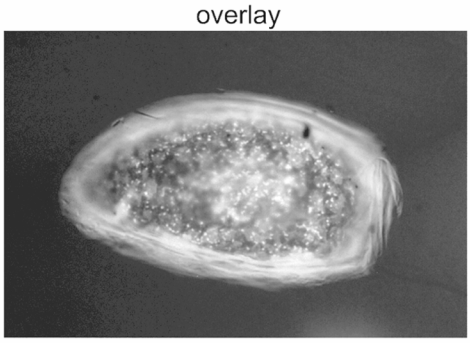
Figure 2 a-c: BUP-GFP protein fusion localization in onion and *Arabidopsis* cells

Transiently (onion) and stably (*Arabidopsis*) transformed cells were analyzed for localization of the BUP-GFP fusion protein, using wide field fluorescence microscopy. a. Golgi-markers GONST-YFP and ST-RFP, and the cytoplasmic localization of the soluble mGFP5 of the pCAMBIA 1302 construct in onion epidermal cells, b. Localization of BUP-GFP (green) and ST-RFP (red) in onion epidermal cells. Co-localization (yellow) is shown in the overlay c. BUP-GFP localization in fibre-like strands in onion cells. Colour figure on page 175.

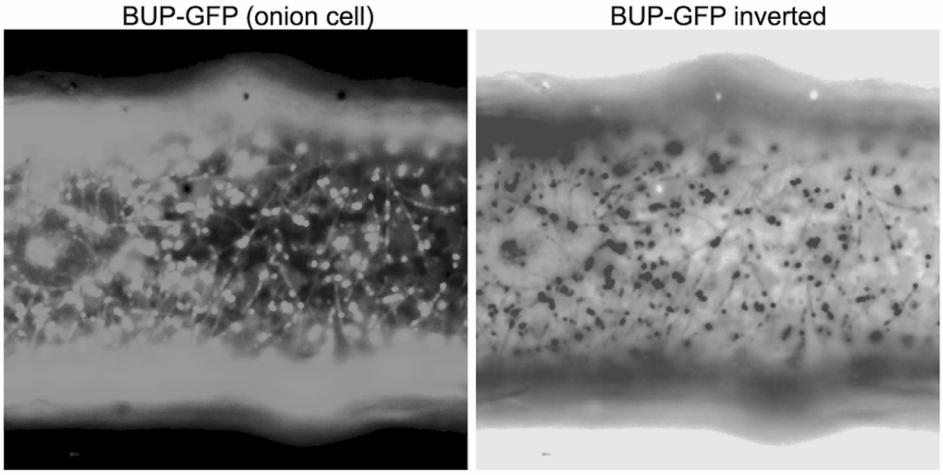

GONST-YFP
ST-RFP
mGFP5

a.


BUP-GFP
ST-RFP


overlay

b.


BUP-GFP (onion cell)
BUP-GFP inverted

c.

BUP-GFP (*Arabidopsis* root cells)

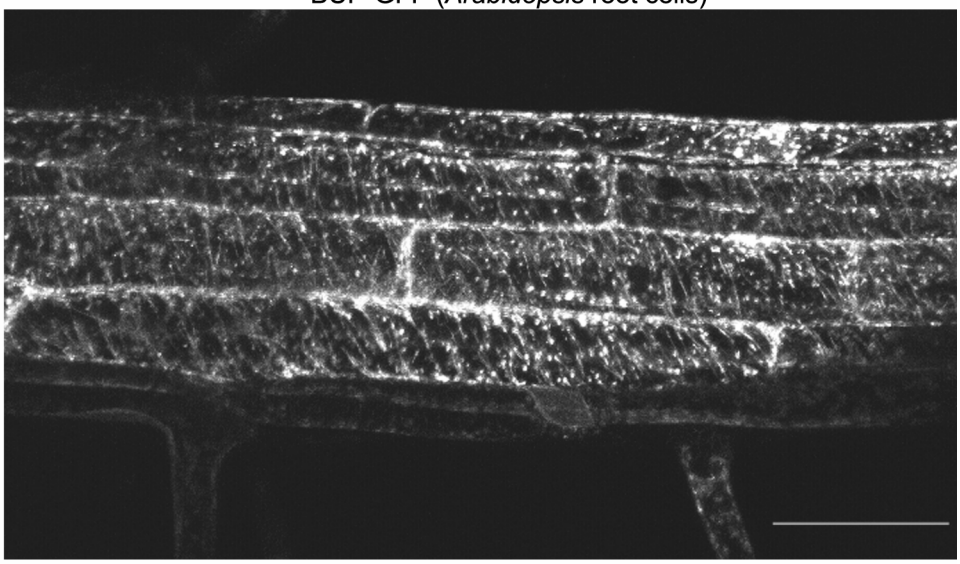
d. 

Figure 2 d: BUP-GFP protein fusion localization in *Arabidopsis* epidermal root cells

The GFP subcellular position might be influenced by post-translation modification of the BUP-GFP fusion protein

Western blot analysis of the BUP-GFP fusion protein was performed to analyse the protein conformation and subcellular localization. Proteins were extracted from onion peel cells and from leaves of *Arabidopsis* transformants showing high expression of *BUP:GFP* (35S::BUP:GFP #7) or *GFP* and from wild-type rosette leaves. The protein extracts were fractionated in a cytoplasmic fraction, containing all soluble proteins of the cytoplasm, an organelle fraction, containing whole organelles, and a microsomal fraction, containing the plasma membranes and organelle membrane-remnants (figure 3). Both in onion and in *Arabidopsis* cells expressing the soluble GFP protein, a protein of about 33 kDa was detected with the GFP-antibody in the cytoplasmic protein fraction alone (black arrowheads figure 3). The predicted size of the soluble mGFP5 protein is 28 kDa, and posttranslational modification of the protein backbone could explain the small increase in protein size in planta and the presence of two mGFP isoforms in the *Arabidopsis* leaves. Western blot analysis of the BUP-GFP fusion protein in both onion and *Arabidopsis* cells showed that a substantial proportion of the fusion protein was cleaved, and a 33 kDa soluble GFP was detectable in the onion and *Arabidopsis* cytoplasmic protein fractions and in the *Arabidopsis* total protein extract (empty arrowheads). A 55 kDa protein was detected in both the organelle and membrane fraction, corresponding to the BUP-GFP fusion protein with the predicted size of 46.7 kDa (arrows). In onion cells, this fusion protein also seemed to be present in the cytoplasmic protein fraction, though this protein appeared to be

slightly bigger than that of the organelle and membrane fractions (figure 3a). Apparently, the N-terminal BUP domains present in the 55 kDa fusion protein detected by the GFP antibody seemed to target the fusion protein to the membrane. However, when the fusion protein was modified by posttranslational cleavage and the GFP protein was released from the transmembrane domain, this GFP became soluble and thus detectable in the cytoplasmic fraction.

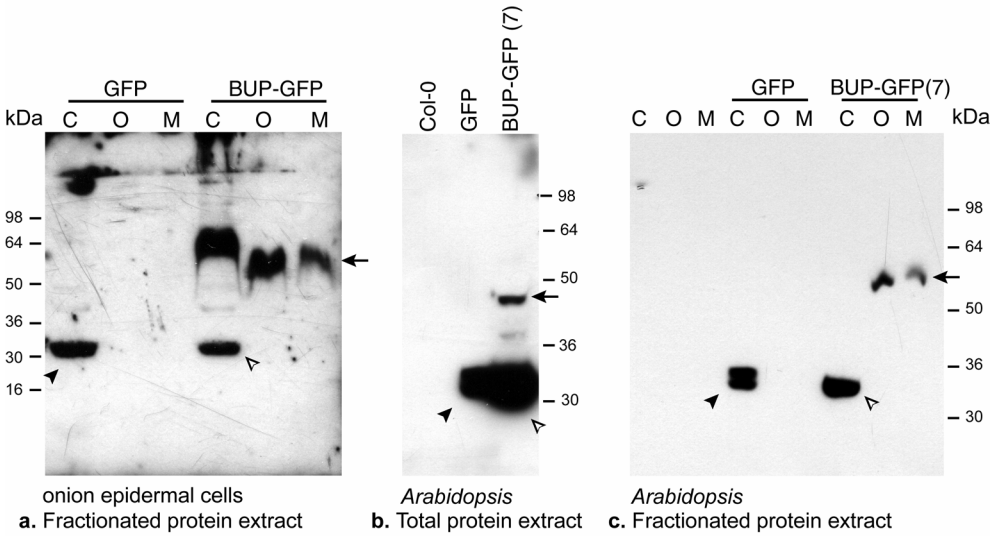


Figure 3: Detection of GFP and BUP-GFP proteins by western blot analysis.
Immuno-blot of a. onion epidermal peel cells and b&c. *Arabidopsis* rosette leaves. The total protein extract was fractionated in a cytoplasmic fraction (C), an organelle fraction (O) and a microsomal fraction (M). Blots were incubated with an anti-GFP antibody. Arrowheads: ~33 kDa soluble mGFP, arrow: ~46.7 kDa, the predicted size of the BUP-GFP fusion protein

BUP immuno-localization and western blot analysis

Antibodies were raised against an amino-terminal or a carboxy-terminal peptide of the BUP protein, N-BUP and C-BUP respectively (figure 4). Each peptide was linked to the carrier Keyhole Limpet Hemocyanin (KLH) by the formation of MBS mediated disulfide bonds. Chicken of which the preimmune sera did not show any background signal on western blot or immuno-electron microscopic sections (data not shown) were immunized with one of the two conjugates. As BUP mRNA was shown to be ubiquitously present in the sporophytic tissues as well as in mature pollen (Chapter 5), western blot analysis was performed on protein extracts of leaves. Unfortunately, after incubation of the protein blots with the N-BUP antiserum multiple protein bands were detected, while no signal above the background level was detected using the C-BUP anti-serum (figure 5a and data not shown). The signal upon incubation with the N-BUP antiserum was approximately 120 kDa, the predicted size for the native BUP protein backbone, but a high

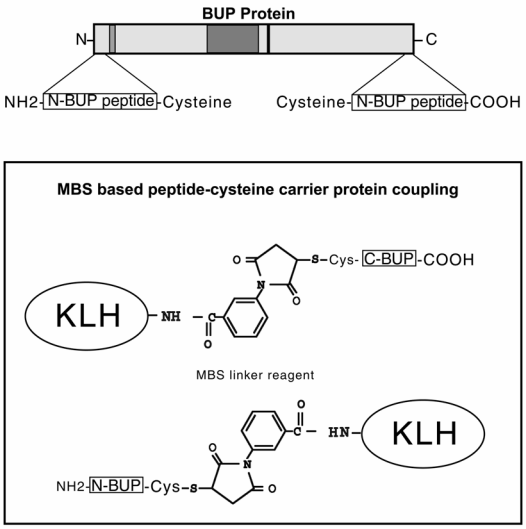


Figure 4: BUP peptides for antibody synthesis
The 20 amino-acid long peptides were linked to the KLH carrier protein by the formation of disulfide bridges between the Cysteine residue (added for this purpose) and the MBS linker reagent bound to KLH.

background level was present. The presence of anti-KLH antibodies in serum have been shown to cause such background problems before (Höglund *et al.,* 2002). To test the antibody specificity, the carrier protein KLH and the two peptide-KLH conjugates were spotted on membrane and incubated with the chicken anti-sera. Figure 5b shows that both anti-sera reacted not only with their specific peptide-conjugate, but also with KLH and the other peptide-KLH conjugate, indicating that the sera were indeed cross-reacting with the KLH carrier protein. When the anti-sera were pre-incubated with KLH the N-BUP and C-BUP anti-sera did not react with the KLH spot, showing that the anti-KLH antibodies were successfully blocked. However, the N-BUP antiserum blocked with KLH still reacted with the C-BUP-KLH conjugate and *vice versa*, indicating that this cross-reaction could not be due to KLH-specific antibodies. As the peptides do not show any homology on amino acid level, the two anti-sera thus seem to cross-react with either the cysteine residue added to the peptide or the MBS linker reagent, both used to mediate the formation of the disulfide bond between the peptide and the carrier protein (figure 4).

To eliminate the aspecific reaction of the N-BUP antiserum, the N-BUP antibody was purified by affinity purification to the N-BUP peptide (without carrier). The active concentration of the N-BUP specific (N*-BUP) antibody was strongly reduced by this purification step. Western blot analysis using a large concentration of the N*-BUP antibody gave variable results: no or faint signals, or detection of a

protein in wild-type *Arabidopsis* plants that was approximately 32 kDa (figure 5c). As the BUP-GFP fusion protein contains the N-terminal peptide against which the N*-BUP antibody was raised, it was expected that western blot analysis of *35S::BUP:GFP Arabidopsis* transformants with this purified antibody would show the presence of the 55 kDa fusion protein. Although two additional proteins were detected in BUP-GFP plants compared with wild-type plants, BUP-GFP plants otherwise showed the same detection pattern on blots as *35S::GFP* transformants, indicating that the N*-BUP antibody did not recognise the N-BUP peptide.

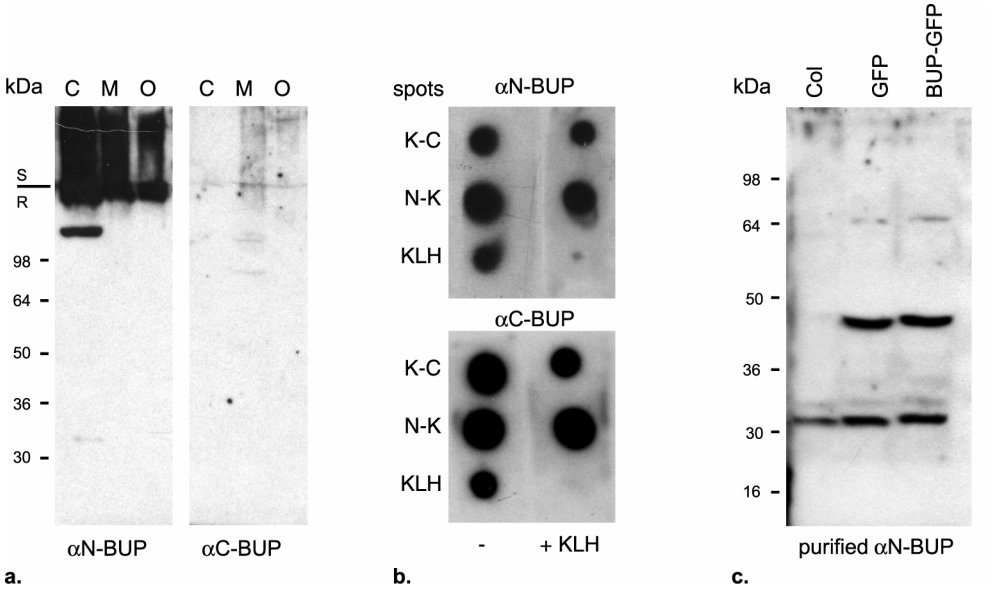


Figure 5: Western blot analysis using BUP peptide antibodies
a. Immuno-blot using BUP peptide antibodies. *Arabidopsis* Col-0 leaf protein extract fractionated in a cytoplasmic fraction (C), microsomal fraction (M) and organelle fraction (O). b. peptide spots incubated with the antisera and with the antisera blocked with KLH. c. Immuno-blot using the affinity-purified N-BUP antibody. Total protein extracts of wild-type *Arabidopsis* and *35S::GFP* and *35S::BUP:GFP Arabidopsis* transformants

Immuno localization

For immuno-localization, leaf tissue and roots were chemically fixed and pollen tubes were fixed by freeze fixation. The tissues were embedded in plastic, sectioned and incubated with the N-BUP or C-BUP antiserum or with the N*-BUP antibody. Similar to results observed on western blots, the unpurified N-BUP or C-BUP anti-sera were aspecific, resulting in high background labelling. When the sections were more severely blocked for aspecific binding, hardly any gold particles were detectable. Similarly, the purified N*-BUP antibody gave no significant immuno-gold labelling of plant tissues (data not shown). To overcome this problem,

commercially available GFP antibody was used for immuno-localization of the BUP-GFP fusion protein in roots and pollen tubes of the *35S::BUP:GFP* or *35S::GFP* and *LAT52::BUP:GFP* or *LAT52::GFP Arabidopsis* transformants, respectively. Though these transformants showed bright GFP fluorescence when analysed with the fluorescence microscope, immuno-localization of the soluble GFP protein did not result in a significant labelling with gold particles. In the *35S::BUP:GFP* epidermal root cells, a few gold particles were located on the Golgi bodies, though due to the low amount of signal this was not considered significant (data not shown).

Discussion

The domain structure of BUP strongly resembles that of Golgi-resident glycosyl transferases (Keegstra *et al.,* 2001), with an amino-terminal cytoplasmic tail, a transmembrane domain and a large carboxy-terminal luminal region in which the glycosyl transferase domain and the activated nucleotide-binding site most likely indicate the catalytic site of the protein. BUP has been shown to be essential for pollen germination and pollen tube growth, as mutant pollen grains burst during or shortly after pollen germination (Chapter 4). This may suggest that BUP is involved in cell wall synthesis, and as such might have an effect on the rigidity and flexibility of the cell wall. BUP does not contain the proposed Golgi-retention signal S/T-X-E/Q-R/K near the transmembrane domain (Bendiak *et al.,* 1990), though this does not mean it is not localized in the Golgi, as many glycosyl transferases do not appear to have a signal peptide (Young, 2004). The length of the transmembrane domain however, might give an indication for the subcellular localization. BUP has a predicted transmembrane domain of 22 amino acids which, according to the lipid-mediated sorting model for Golgi-localized glycosyl transferases, is in agreement with localization in the Golgi. Most Golgi-resident single pass transmembrane proteins have a hydrophobic domain of 17 to 20 amino acids long, whereas plasma membrane proteins contain a transmembrane of at least 23 amino acids (Watson *et al.,* 2001; Brandizzi *et al.,* 2002). The relatively long BUP domain of 22 amino acids could suggest that this protein is localized preferentially in the trans Golgi rather than the cis or median cisternae of the Golgi.

Experimental analysis of the subcellular localization of BUP to test the Golgi-localization hypothesis was performed using GFP fusion proteins, western blot analysis and immuno localization using BUP and GFP antibodies. In the chimeric BUP-GFP fusion protein, GFP was fused to an amino-terminal part of BUP containing the relevant domains for Golgi localization (reviewed in Gleeson, 1998 and Gomord *et al.,* 1999). Co-localization with the Golgi marker ST-RFP showed that BUP-GFP was labelling Golgi bodies, but also strand-like structures that could be part of the cortical cytoskeleton. Previous work extensively demonstrated that the plant Golgi moves on an actin/ER network within the cytoplasm (Boevink *et al.,* 1998; Nebenführ *et al.,* 1999; Baldwin *et al.,* 2001 and reviewed in Hawes *et al.,* 2005). From our localization experiments however, it does not seem that BUP-GFP fusion protein is localized to the ER, rather it may be associated with actin filaments or cortical microtubules. If this is true, not all GFP fluorescing proteins were membrane associated. Western blot analysis of expressed BUP-GFP showed that some of this fusion protein had been cleaved to produce a soluble form of GFP, and this soluble GFP therefore does not reflect BUP localization but instead may aspecifically label actin or microtubules.

In pollen tubes, there is an increase in the number of Golgi bodies present behind the secretory vesicle region, or clear region, of the pollen tube (Derksen *et al.,* 2002 and Chapter 2). Glycosylated cell wall components synthesized in these Golgi bodies are secreted from the secretory vesicles at the pollen tube tip. If BUP is targeted to the Golgi, an increase in BUP-GFP fusion protein accumulation is expected at the region flanking the tip of the pollen tubes. Analysis of pollen tubes of *LAT52::BUP:GFP* transformants, however, did not result in accumulation of GFP signal near the tip. Presumably, proteolytic cleavage of the fusion protein, as described for onion and *Arabidopsis* cells, also occurred in the pollen tubes, resulting in the observed predominant labelling of cytoplasmic components including the cytoskeleton.

Glycosyl transferases can undergo proteolytic cleavage at the stem region within the Golgi lumen (Weinstein *et al.,* 1987; Masri *et al.,* 1988; Homa *et al.,* 1993; reviewed in Young, 2004). When this occurs, the transmembrane domain and its flanking sequences will remain in the Golgi membrane, while the soluble glycosyl transferase hypothetically can remain in the Golgi lumen, can be transported to the cytoplasm or can be excreted. In plants, the uridine diphosphate (UDP) glycosyltransferases, which also belong to the glycosyl transferase 1 family, are generally thought to be soluble proteins, localized within the cytoplasm (Ross *et al.,* 2001). Extracellular secretion of glycosyl transferases has been found mostly in studies on animal glycosyl transferases, in which soluble isoforms were found in the media of cultured cells or in body fluids (reviewed in Cho *et al.,* 1997; Breton *et al.,* 2001; El-Battari *et al.,* 2003), and no such reports exist for plants. Cleavage of the BUP-GFP fusion protein thus could either be an artefact, or it could indicate the presence of an actual cleavage signal in the stem region of BUP. Hypothetically, soluble extracellular glycosyl transferases in plants could modify the glycosylation of cell wall components that are already located within the cell wall, though the activated-sugar substrate would have to be present in the extracellular matrix as well. Known extracellular enzymes that are involved in cell wall modification are expansins, glycosylases, xyloglucan endo-transglycosylases (XET) and pectin methylesterases. All these enzymes seem to be directly or indirectly involved in ‘loosening’ of the cell wall, which enables cell expansion and cell growth (McQueen-Mason and Cosgrove, 1995; Cosgrove, 2000; Pezzotti *et al.,* 2002; Nishitani, 1997; Fischer and Bennett, 1991; Liu and Berry, 1991; Carpita *et al.,* 1996). In tobacco, the arabinogalactan protein TTS is glycosylated, forming a gradient in glycosylation from the top of the style towards the ovule (Wang *et al.,* 1993). This gradient is implicated to be involved in pollen tube guidance to the ovary, and differential glycosylation thus might play an important role in pollen-pistil interactions.

In western blot analysis of the wild-type BUP protein using peptide antibodies, multiple proteins were detected. However, these were not the result of proteolytic

cleavage of the BUP protein, but were proteins aspecifically reacting with the antibody. In fact, this antibody apparently did not recognise the N-terminal part of BUP, because it could not detect the 55 kDa BUP-GFP fusion protein expected in the transformed plants. Also immuno-localization of BUP or the BUP-GFP fusion protein in *Arabidopsis* transformants, using the N-BUP antibody and the GFP antibody respectively, did not give a signal high enough to be able to localize the BUP protein within the plant cells.

Though many of the experiments performed to analyse the subcellular localization of BUP gave inconclusive results, the co-localization of the BUP-GFP fusion protein with the ST-RFP Golgi-marker and its strong association with membranes clearly indicate that BUP is targeted to the Golgi apparatus. Whether the native BUP protein will be subject to proteolytic cleavage, like the fusion protein, remains to be analysed. This Golgi localization, together with the structural features typical for glycosyl transferases suggest that BUP might indeed be a functional glycosyl transferase.

Chapter 7

Concluding Remarks

Segregation distortion analysis, a forward genetics tool for the identification of male gametophytic and essential sporophytic genes

To analyze genes involved in sexual reproduction in plants, mutants with a defect in pollen function were isolated from a T-DNA insertional mutant population by screening for segregation distortion. Normally, a single, hemizygous T-DNA insertion leads to a 3:1 segregation of the antibiotic resistance gene in the offspring. Plants with a sex-specific gametophytic lethal mutation however, fail to transmit the T-DNA insertional mutation through the affected gamete, which will result in a segregation of 1:1 resistant to sensitive seedlings (Feldmann *et al.,* 1997; Howden *et al.,* 1998). In this thesis I present the identification and characterization of the mutant line TJ995, in which T-DNA transmission through pollen was severely reduced to only 2% of the wild-type transmission efficiency, while female T-DNA transmission was unaffected (Chapter 3). Gene At5g04480 was isolated as the underlying mutated gene, and was named *BURSTING POLLEN (BUP)*, as mutant pollen show a burst phenotype during pollen germination *in vitro*.

Gene expression analysis showed that *BUP* was constitutively expressed at very low levels: expression in pollen could only be detected with RT-PCR analysis, not with (the less sensitive) micro-array analysis (Chapter 5). Apparently, some genes involved in pollen development and function, like *BUP*, are expressed at a very low level. As such, these genes are less likely to be identified as potentially important genes using a transcriptomic approach like micro-array analysis.

Though the *bup* mutation is residually transmitted through pollen, no homozygous *bup* mutant plants could be identified, indicating that the homozygous mutation of this gene might be embryo-lethal. In fact, the majority of the male gametophytic mutants described in literature that do show residual T-DNA transmission do not give rise to homozygous mutant progeny (Introduction, table 1), suggesting that these mutants are also embryo-lethal. Embryo-lethal mutations are often recessive mutations in genes that are either embryo-specific or essential for sporophytic development, including so called housekeeping genes. Obviously, no homozygous mutant plants are formed due to the defect in seed development, and embryo-lethal mutants have been isolated from mutant populations by screening either for a visible defect in seed development or for a distorted segregation of 2:1 instead of 3:1 (Errampalli *et al.,* 1991; Castle *et al.*, 1993). When these recessive mutations also cause a defect in the haploid male gametophyte, the embryo-lethality phenotype may be difficult to identify, as none or only a small fraction of homozygous embryos will be formed. Hence, many mutations in essential sporophytic genes might be hidden in gametophytic mutants showing a 1:1 segregation, in addition to mutations of the 2:1 segregating plants.

In this era of genomics and reverse genetics, in which the American National Science Foundation and the *Arabidopsis* community have set themselves the task to determine the function of all 25,498 *Arabidopsis* genes by the year 2010 (http://*www.nsf.gov/pubs/2004/nsf04502/nsf04502.htm*), forward genetics tools like segregation distortion analysis still prove to be indispensable for the identification of both male gametophytic genes and housekeeping genes.

Pollen fitness and pollen expression of sporophytic genes

Pollen gene expression analyses have shown that genes expressed in pollen can be pollen-specific or can also be expressed in sporophytic tissues. The pollen-specific genes generally show very high expression levels and code for proteins that are predominantly involved in signalling and in cell wall synthesis (Honys and Twell, 2003;2004; Becker *et al.,* 2003; Lee *et al.,* 2003). In contrast, the group of genes that are both gametophytically and sporophytically expressed (which constitutes ~90% of all genes expressed in pollen) show a much lower expression level and these genes functionally reflect the sporophytic transcriptome (Honys and Twell, 2003; 2004; Becker *et al.,* 2003).

Genes expressed in pollen undergo strong natural selection as an excess amount of pollen present on a stigma leads to competition of the fittest pollen to fertilize the limited number of embryo sacs (Mulcahy *et al.,* 1996). If only pollen-specific genes would have an effect on pollen tube growth and thus on pollen fitness, this selection would not be a benefit for the fitness of the sporophyte, which from an evolutionary perspective would not make much sense (Mulcahy *et al.,* 1996). Perhaps this is why so many sporophytically expressed genes are also expressed in pollen: to enable the selection of sporophytically important genes, like housekeeping genes, through pollen fitness.

Though *BUP* is expressed only at a low level in pollen, mutant pollen show a severe reduction in fitness. *BUP* apparently is important for cellular processes affecting pollen function and fitness. As *BUP* is likely to be important for sporophytic function as well, selection for the functional allele in pollen ensures the presence of a functional allele during sporophyte development. Like other embryo-lethal male gametophytic mutants such as the mutant of AtCSLA7 (Goubet *et al.,* 2003), *bup* does not have a defect in the female gametophyte. If we take the hypothesis of sporophytic gene selection in pollen one step further, one could speculate that these sporophytic genes do not need to have such an essential role in the embryo sac, as embryo sacs do not undergo competition to the same extent as pollen.

Splicing of the *BUP* transcript is conserved through evolution

Protein homology analysis has shown that BUP has three homologous proteins present in the GenBank database: one *Arabidopsis* homologue, BUP-like (BPL, At4g01210 gene product) and two rice proteins (Chapter 3). The progenitors of the monocot rice species and the dicot *Arabidopsis* species have diverged from a common ancestor 150-200 million years ago (Bennetzen, 2002). Therefore, the level of protein homology and the strong conservation of the exon structure of the encoding genes through evolution suggest that these BUP homologues are important for the overall plant function.

Incomplete penetrance of the *bup* mutation: quantitative defect versus molecular nature of the mutation

The two mutant alleles of *BUP*, *bup-1* and *bup-2*, both showed a severely reduced male T-DNA transmission efficiency of 2%. In both mutants, large insertions of the (multiple) T-DNA copies are present which likely cause a defect in transcription, though truncated proteins might still be formed from the first part of the mRNA. Northern blot and western blot analyses to investigate the molecular nature of the mutations unfortunately did not satisfactorily answer this question. Considering the fact that *bup-1/+* and *bup-2/+* showed the same level of limited male transmission, although the T-DNA insertions in the two mutants were inserted at different places within the gene, it is likely that no mutant protein is formed at all. Therefore, the residual T-DNA transmission through pollen must be caused by other factors, like partial gene redundancy or environmental factors.

Gene expression analysis of *BUP* and *BUP-like (BPL)* showed that they are not expressed at the same time during pollen development. *BUP* is expressed in mature pollen, while *BPL* is expressed earlier during pollen development (Chapter 5). This, together with the relatively low identity (34%) between the two homologous proteins, suggests that *BUP* and *BPL* are not functionally redundant in pollen. Environmental factors on the other hand, may play some role in the fitness of the mutant pollen, as we observed bursting of germinating pollen *in vitro*, but not *in vivo* on the stigma, and the fitness of the mutant pollen was sensitive to competition by wild-type pollen tubes (Chapter 4).

BUP might be involved in pectin synthesis and cell wall strength

The domain structure of the BUP protein strongly resembles that of a glycosyl transferase family 1 member, being a type II transmembrane protein with a glycosyl transferase 1 domain and a GTP/ATP binding site (Chapter 3). Though *BUP* and *BPL* were not among the 121 *Arabidopsis* glycosyl transferase 1 proteins

annotated in the Cazy database (Coutinho and Henrissat, 1999), protein structural analysis has shown that these genes belong to the glycosyl transferase 1 family (Egelund *et al.,* 2004). Protein localization studies (Chapter 6) indicated that BUP might be localized in the Golgi apparatus, like most transmembrane glycosyl transferases (Keegstra and Raikhel, 2001), though cleavage of the protein-fusion construct and non-specific reactions of the antibodies made the results difficult to interpret.

bup/+ pollen showed bursting of the pollen during *in vitro* pollen germination, which could be explained by a defect in the structure of the pollen cell wall during pollen germination (Chapter 4). Consistent with a function in cell wall synthesis, micro-array data (Genevestigator database) showed the highest expression level of BUP in the stem, a tissue type in which the rigidity of the cell wall is obviously an important factor, while the highest *BPL* expression was found in *Arabidopsis* suspension cells treated with the herbicide isoxaben, a strong inhibitor of the cellulose synthesis (Chapter 5). Suspension cells habituated to isoxaben can compensate for the disruption of cellulose synthesis by the synthesis of other cell wall components like pectin (Manfield *et al.,* 2004).

The pollen cell wall consists of the intine layer that is mainly composed of pectin and cellulose, and the exine layer that is made up of sporopollenin. During pollen germination, accumulation of pectic, callosic and cellulosic material at the site of pollen tube emergence causes thickening of the intine, forming a ‘germination plaque’ (Chapter 4). Pectin and hemicelullose are synthesized in the Golgi apparatus. Single membrane-spanning glycosyl transferases are required for the synthesis of the homogalacturonan pectin backbone and side chains. Perturbation of pectin transport by monensin, brefeldin A and cytochalasin D during pollen germination results in the arrest of pollen tube growth (Geitmann *et al.,* 1996), indicating that pectin is an essential component within the pollen tube wall. When highly methyl-esterified pectin is excreted by the Golgi-derived secretory vesicles (Gibeaut and Carpita, 1994) in the intine layer and at the tip of the pollen tube, this pectin is rather flexible and can enable cell wall expansion. During pollen germination and pollen tube growth, the pectin in the wall is de-esterified and can cross-link with calcium ions to form an inflexible pectin gel, increasing the strength of the cell wall (Jarvis, 1984; Carpita and Gibeaut, 1993). The bursting phenotype of *bup* pollen *in vitro* is very similar to the bursting pollen tubes of the *Arabidopsis vanguard* mutant (Jiang *et al.,* 2005). *VANGUARD* codes for a pectin methylesterase which is suggested to play a role during pollen tube growth by increasing cell wall strength through de-methylesterification of methyl-esterified pectin. *VANGUARD* is expressed specifically in pollen and pollen tubes, and due to residual T-DNA transmission homozygous plants were formed. Though no burst pollen tubes were seen *in vivo*, the *vgd1* pollen tubes grow slower than wild-type pollen tubes, and were only able to fertilize embryo sacs at the top of the ovary.

The authors suggested that the hydration expansion of pollen *in vivo* might be milder or more controlled as compared to the *in vitro* conditions. BUP seems to act earlier than VANGUARD, as *in vitro bup* pollen burst prior to the formation of pollen tubes. This is consistent with a function of BUP during pectin synthesis in the Golgi apparatus rather than modification of the pectin structure within the cell wall, and BUP might be one of the glycosyl transferases involved in the transfer of sugar residues as side chains onto the pectin backbone.

Conclusions

Taken together, the similarity of BUP to glycosyl transferase 1 proteins, the bursting pollen phenotype *in vitro*, the high expression level in tissues that need strong cell walls like the stem and the similarity to another mutant affected in pectin metabolism within the pollen tube cell wall all suggest a role for BUP in the synthesis of pectin, or hemicellulose, and thus in the strength of pollen and pollen tube walls. As *BUP* is also expressed in the vegetative tissues, and no homozygous mutants were found, BUP could also be essential for the cell wall composition of the sporophyte.

Chapter 8

Summary

Samenvatting

Summary

Sexual plant reproduction, in particular pollen tube tip growth and the signalling between pollen and pistil have been studied for many decades, mainly in model species with large, bicellular pollen like tobacco and lily. A significant increase in the knowledge of genes involved in these processes however, was made using *Arabidopsis thaliana*, the plant of choice for genetic and molecular studies, by screening mutant populations and by genome-wide analysis of genes expressed in pollen using micro-arrays. To compare the cellular organization of 3the tricellular *Arabidopsis* pollen tubes with those of the established bicellular pollen tube models the ultrastructure of *in vitro* grown *Arabidopsis* pollen tubes was analyzed. *Arabidopsis* pollen tubes have similar growth *in vitro* compared to tobacco and lily pollen tubes, though the germination frequency and speed of pollen tube growth was much lower (Chapter 2).

The main focus of this thesis is the genetic, molecular and cellular characterization of a male gametophytic mutant, found in a T-DNA insertional *Arabidopsis* mutant population by segregation distortion analysis. The mutant line TJ995 showed a severe defect in male T-DNA transmission efficiency, with only 2% of the mutant pollen tubes being able to achieve fertilization (Chapter 3). No defect through the female gametophyte was found. Despite the complex T-DNA insertion pattern in this line, At5g04480 was identified as the affected gene. This was confirmed by segregation analysis of an independent allelic mutant and by molecular complementation of the two mutants (Chapter 3).

At5g04480 was named *BURSTING POLLEN (BUP),* as mutant phenotypic analysis showed bursting of the mutant pollen during pollen germination and pollen tube growth *in vitro* (Chapter 4). This bursting pollen phenotype suggested a defect in the strength of the pollen wall that might be related to pectin present in the intine layer. *In vivo,* also mutant pollen tube growth was affected, as is shown by the increase in T-DNA transmission in the absence of competition by wild-type pollen tubes.

BUP encodes for a type II transmembrane protein showing similarity with family 1 glycosyl transferases, and has one *Arabidopsis* protein homologue (BUP-like, BPL) and two homologous rice proteins. The strong conservation of the exon-boundary structure of these proteins through evolution suggests that BUP and its homologues are important proteins (Chapter 3). Gene expression analysis showed that *BUP* and *BPL* both were expressed at low levels in pollen and in vegetative tissues. In pollen, *BPL* is expressed early during pollen development, while *BUP* is expressed in mature pollen (Chapter 5), suggesting that *BUP* and *BPL* have different functions within the male gametophyte. BUP protein localization was analyzed by protein-fusion analysis, immuno-localization and western blot analysis (Chapter 6). Most glycosyl transferases with a single transmembrane domain are

localized in the Golgi apparatus. The co-localization of the BUP-GFP fusion protein with a Golgi-marker in onion epidermal cells suggested that BUP was indeed targeted to the Golgi apparatus, however, this could not be conclusively confirmed by immuno localization and western blot analysis due to instability of the fusion protein and technical difficulties with the antibodies used.

In conclusion, the defect in pollen wall strength of *bursting pollen* and the phenotypic similarity of the *bup* mutants with other pectin pollen mutants suggest that BUP might function as a glycosyl transferase involved in the synthesis of pectin. As *BUP* is expressed in the sporophyte, and no homozygous mutants could be identified, it is likely that the homozygous *bup* mutant is embryo-lethal, and that this gene plays an important role in cell wall synthesis during sporophyte development in addition to its role in pollen germination and pollen tube growth.

Samenvatting

Onderzoek naar het proces van seksuele voortplanting in planten, en met name pollenbuisgroei en de celcommunicatie tussen pollen en pistil, werd oorspronkelijk uitgevoerd in soorten met grote, bicellulaire pollenkorrels zoals tabak en lelie. Dit heeft veel informatie opgeleverd over de ultrastructuur van de pollenbuis *in vitro*, de regulatie van het proces van tipgroei in pollenbuizen en over het proces van self-incompatibiliteit. Met de komst van *Arabidopsis thaliana* als model systeem voor genetisch en moleculair onderzoek was het mogelijk om gebruik te maken van mutantenpopulaties en gen-expressie onderzoek met behulp van micro-array analyse. Hierdoor is gedurende het laatste decennium ook steeds meer bekend geworden over de genen die betrokken zijn bij deze processen. In tegenstelling tot pollen van tabak en lelie zijn rijpe *Arabidopsis* pollenkorrels tricellulair. In Hoofdstuk 2 wordt de ultrastructuur van wildtype *Arabidopsis* pollenbuizen *in vitro* beschreven en wordt deze vergeleken met de bekende pollenbuiskarakteristieken van soorten met bicellulaire pollen. Electronenmicroscopie toonde aan dat de ultrastructuur van *Arabidopsis* pollenbuizen vrijwel hetzelfde was, maar dat de groeisnelheid van deze pollenbuizen *in vitro* beduidend lager is (Hoofdstuk 2).

In dit proefschrift wordt de identificatie en de moleculaire karakterisatie van een mannelijk gametofytische mutant beschreven. Deze mutant, lijn TJ995, is gevonden in een T-DNA insertie mutantenpopulatie met behulp van segregatie distortie analyse. Na zelfbestuiving bezat slechts de helft van de nakomelingen van deze lijn het antibiotica resistentie-gen dat op het T-DNA ligt (1:1 resistente:sensitieve kiemplantjes), in plaats van de verwachte 3:1 verhouding na normale overerving van het T-DNA volgens de wetten van Mendel (Hoofdstuk 3). Reciproke bestuivingsproeven met wildtype planten hebben aangetoond dat maar 2% van de gemuteerde pollenbuizen de eicel kan bevruchten, terwijl de vrouwelijke transmissie van het T-DNA naar de volgende generatie normaal was. Moleculair onderzoek naar het aantal T-DNA kopieën in lijn TJ995 toonde aan dat er meerdere T-DNA inserties in het genoom aanwezig zijn. Het mannelijk gametofytische defect bleek veroorzaakt te zijn door de mutatie van gen At5g04480, wat onomstotelijk is aangetoond door segregatie distortie analyse van een onafhankelijke mutant van dit gen en door moleculaire complementatie van deze twee onafhankelijke mutanten met het wildtype gen (Hoofdstuk 3).

Lijn TJ995 is hemizygoot voor de mutatie en bevat naast gemuteerde pollenkorrels ook wildtype pollenkorrels. *In vitro* bleek ongeveer de helft van de pollen te barsten tijdens de kieming, de mutant werd daarom *bursting pollen (bup)* genoemd (Hoofdstuk 4). Het barsten van de pollenkorrels tijdens de kieming *in vitro* duidt vermoedelijk op een defect in de wand van de pollenkorrels, waardoor deze niet sterk genoeg is voor de verandering in turgordruk. Het fenotype van *bursting pollen* lijkt sterk op dat van een andere pollen mutant met een defect in de

pectine regulatie in de pollenwand (*vanguard*). Ook in *bup* zou een defect in de synthese of modificatie van het pectine in het intine de verminderde weerstand van de pollenwand kunnen verklaren. Doordat de plant hemizygoot is kon er geen verschil in pollenbuisgroei *in vivo* worden waargenomen. Wel nam de mannelijke transmissie van het T-DNA toe wanneer er geen competitie was door wildtype pollen. Dit wijst erop dat het *BUP* gen niet alleen betrokken is bij de pollenkieming, maar ook bij de groei van de pollenbuis in de pistil.

BUP codeert voor een transmembraan eiwit dat overeenkomsten vertoont met enzymen behorende tot de familie 1 glycosyltransferases. BUP is homoloog aan één ander *Arabidopsis* eiwit (BUP-like, BPL) en aan twee rijst eiwitten. De intron-exon structuur van de vier coderende genen bleek sterk geconserveerd, wat suggereert dat BUP en de andere drie eiwitten een belangrijke functie vervullen in de plant (Hoofdstuk 3). Eiwitlokalisatie van BUP is onderzocht met behulp van eiwit-GFP fusie, immunolokalisatie en western blot analyse (Hoofdstuk 6). De meeste glycosyltransferases met één transmembraan domain bevinden zich in het Golgi-apparaat. Het BUP-GFP fusie-eiwit vertoonde co-lokalisatie met een Golgi-marker in uien epidermaalcellen, dit wijst er op dat BUP inderdaad ook naar het Golgi wordt getransporteerd. De resultaten van de immunolokalisatie en western blot analyse waren echter niet duidelijk genoeg om dit onomstotelijk te kunnen bevestigen.

Analyse van de gen-activiteit van *BUP* en *BPL* liet zien dat beide genen zowel in pollen als in de meeste vegetatieve weefsels tot expressie komen. *BPL* komt echter vroeg tijdens de pollenontwikkeling tot expressie, terwijl *BUP* tot expressie komt in het rijpe stadium (Hoofdstuk 5). Doordat beide genen niet op hetzelfde moment tijdens de pollenontwikkeling actief zijn, betekent dit dat ze hoogst waarschijnlijk niet precies dezelfde functie hebben. Alhoewel de mutatie in *bup* niet volledig penetrant is, en er dus in principe homozygote embryo's gevormd kunnen worden, zijn er geen homozygote mutanten gevonden. Dit geeft aan dat de homozygote *bup* mutatie mogelijk embryo-lethaal is, zeker omdat *BUP* ook tot expressie komt in de vegetatieve plant.

Samenvattend kan worden geconcludeerd dat BUP mogelijk betrokken is als glycosyltransferase bij de synthese of modificatie van pectine in de pollen- en pollenbuiswand. Naast een rol in pollen heeft BUP zeer waarschijnlijk ook een belangrijke functie bij de vorming van de celwand tijdens de ontwikkeling van de sporofiet.

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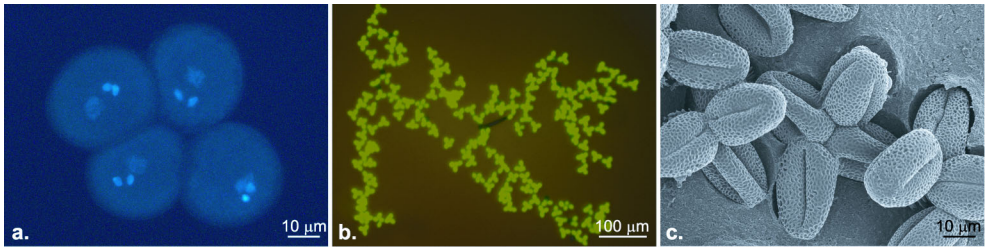
Curriculum Vitae

De auteur van dit proefschrift werd geboren op 6 maart 1977 te Venray. In 1995 behaalde ze het VWO diploma aan het Boschveldcollege te Venray. Datzelfde jaar werd begonnen met de studie biologie aan de Katholieke Universiteit Nijmegen (KUN, nu de Radboud Universiteit Nijmegen). Haar eerste stage werd uitgevoerd op de afdeling Celbiologie van de KUN onder begeleiding van Dr. A.P.R. Theuvenet, en betrof onderzoek naar het effect van aluminium op de groeisignalering van muizenfibroblasten. Haar tweede stage verrichtte ze op het lab van Prof. Dr. H.G. Dickinson van het 'Department of Plant Sciences' aan de Universiteit van Oxford (UK), onder begeleiding van Dr. S.J. Hiscock en externe begeleiding van Prof. Dr. C. Mariani (afdeling Celbiologie van de Plant, KUN). Tijdens deze stage deed ze morfologisch onderzoek naar het systeem van zelf-incompatibiliteit bij de composiet *Senecio squalidus* (glanzend kruiskruid). In januari 2000 werd het doctoraal examen biologie cum laude behaald en in februari van hetzelfde jaar werd zij aangesteld als assistent in opleiding (dat later werd omgezet in junior-onderzoeker) op de afdeling Celbiologie van de Plant van de KUN, onder begeleiding van Prof. Dr. C. Mariani en Prof. Dr. D. Twell van de Universiteit van Leicester (UK). De resultaten van dit promotie onderzoek, waarbij ook een deel van het werk op het 'Department of Biology' in Leicester is uitgevoerd, staan beschreven in dit proefschrift. Naast het begeleiden van twee stagestudenten heeft zij verschillende cursussen gevolgd, waaronder de cursus *Arabidopsis Molecular Genetics* aan het Cold Spring Harbor instituut in de Verenigde Staten (2001), en nam ze deel aan verscheidene internationale congressen, waaronder *de Annual meeting of the Society of Experimental Biology* in Swansea (2002), het *XIII International Conference on Arabidopsis Research* in Sevilla (2002) en het *7th International Congress of Plant Molecular Biology* in Barcelona (2003). Sinds februari 2005 is zij werkzaam als Onderzoeker Celbiologie bij het groentezaden veredelingsbedrijf Nunhems Netherlands B.V. te Haelen.

Colour Figures

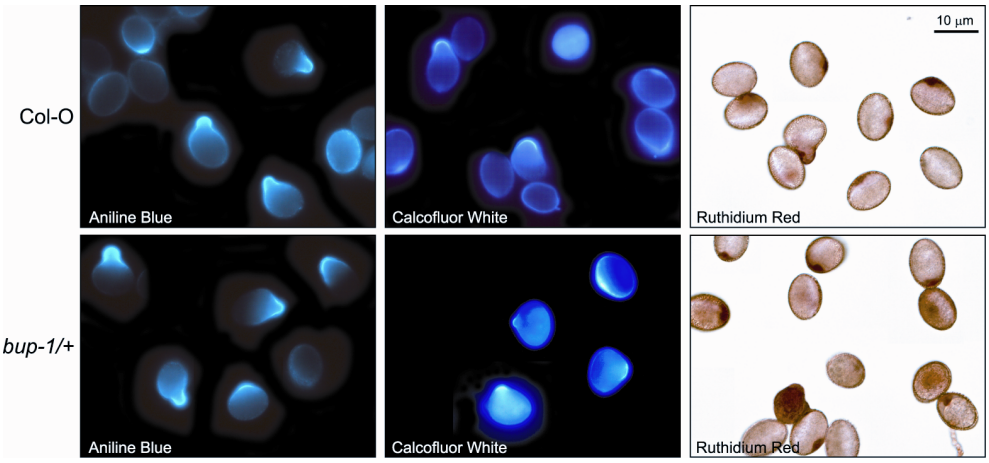
Chapter 4

Chapter 4, figure 1: bup pollen development analysis in bup-1/+ qrt1/qrt1 tetrads



a. DAPI staining showed a normal positioning of the vegetative cell nucleus and the two brightly stained sperm cell nuclei. b. FDA staining of ~100 tetrads showed that all the four pollen grains in the *bup-1/+* tetrads were viable. c. Cryo scanning electron micrograph showing four normally dehydrated pollen with normal exine architecture.

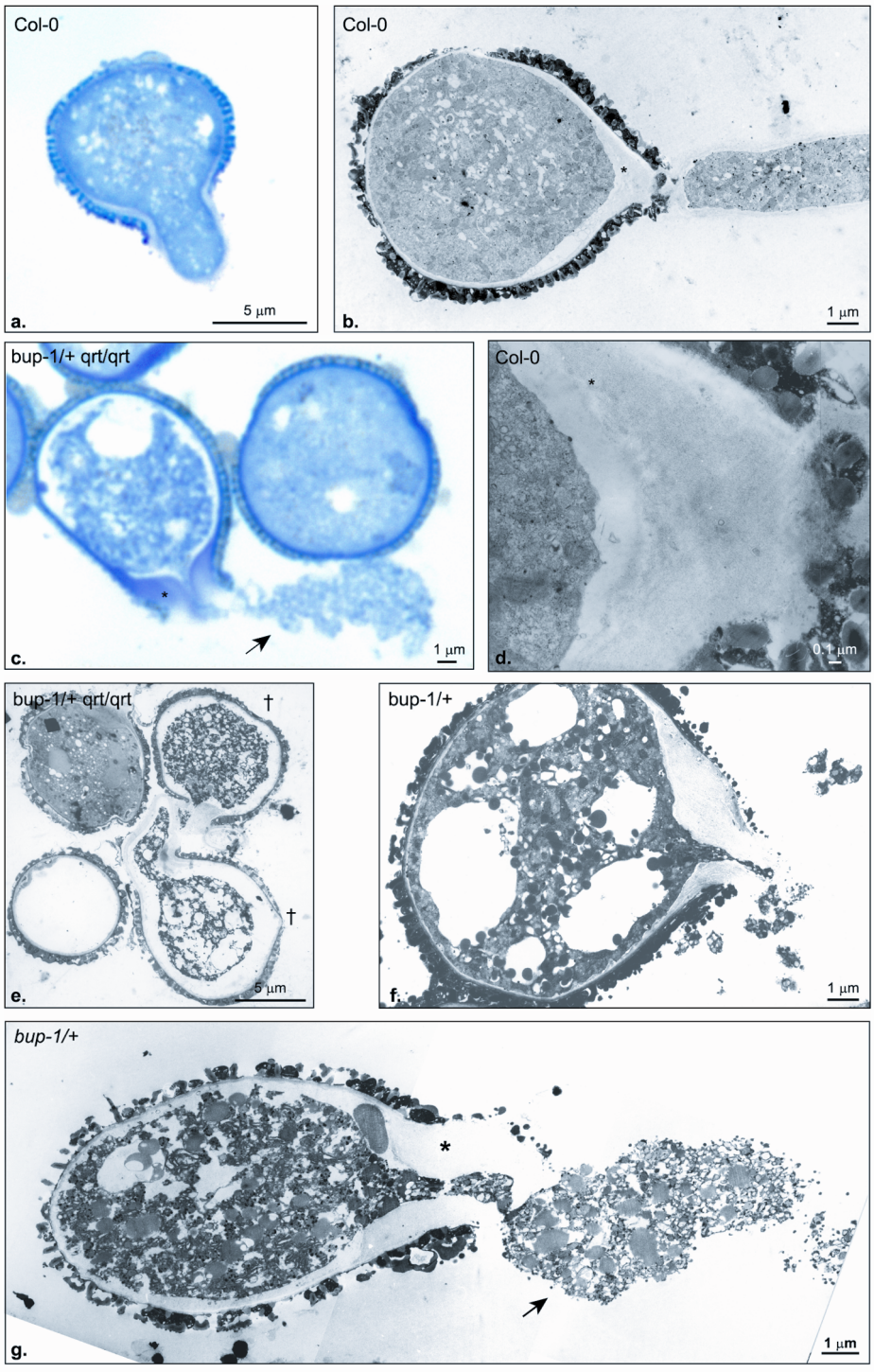
Chapter 4, figure 4: Germination plaque composition



Aniline Blue, Calcofluor White and Ruthidium Red staining of fresh germinating wild-type and *bup-1/+* pollen

Next page:

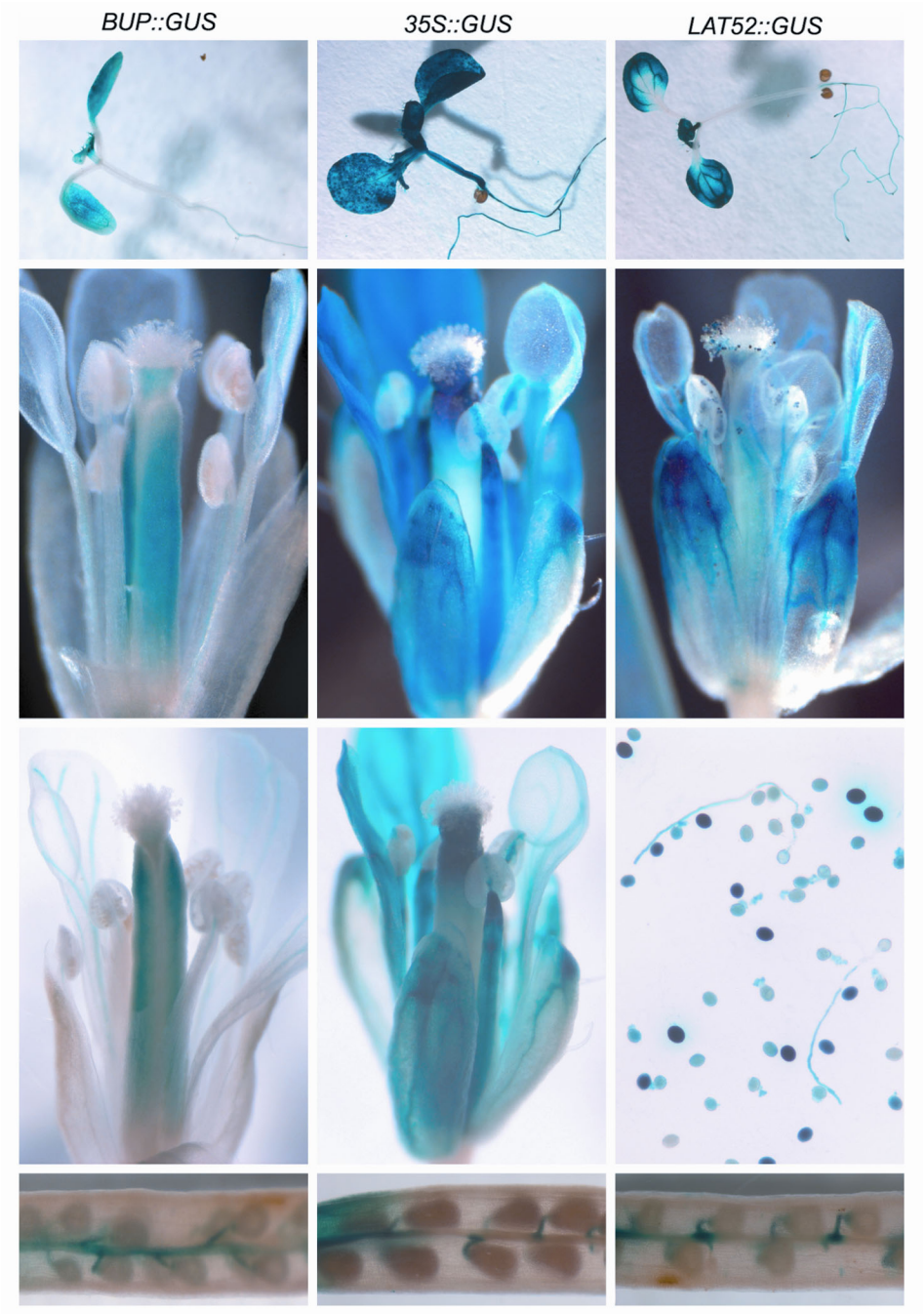
Chapter 4, figure 5: Ultrastructural analysis of *bup-1/+* pollen
Toluidine Blue stained sections (a and c) and transmission electron micrographs (b and d-g) of Col-0, *bup-1/+* and *bup-1/+ qrt1/qrt1* germinated pollen. d, Magnification of plaque in wild type pollen in b. e, †: dead pollen in the tetrad. The other pollen grains have formed a pollen tube (empty pollen) or did not germinate. Arrows: Cytoplasmic protrusions of the mutant pollen, lacking a plasma membrane and cell wall material. Asterisk: germination plaque in wild-type and burst pollen. In the burst pollen, the cytoplasm seemed to have been pushed through a small opening within the plaque.


Col-0
a.
5 µm
Col-0
b.
1 µm
bup-1/+ qrt/qrt
c.
1 µm
Col-0
d.
0.1 µm
bup-1/+ qrt/qrt
e.
5 µm
bup-1/+
f.
1 µm
bup-1/+
g.
1 µm

Colour Figures

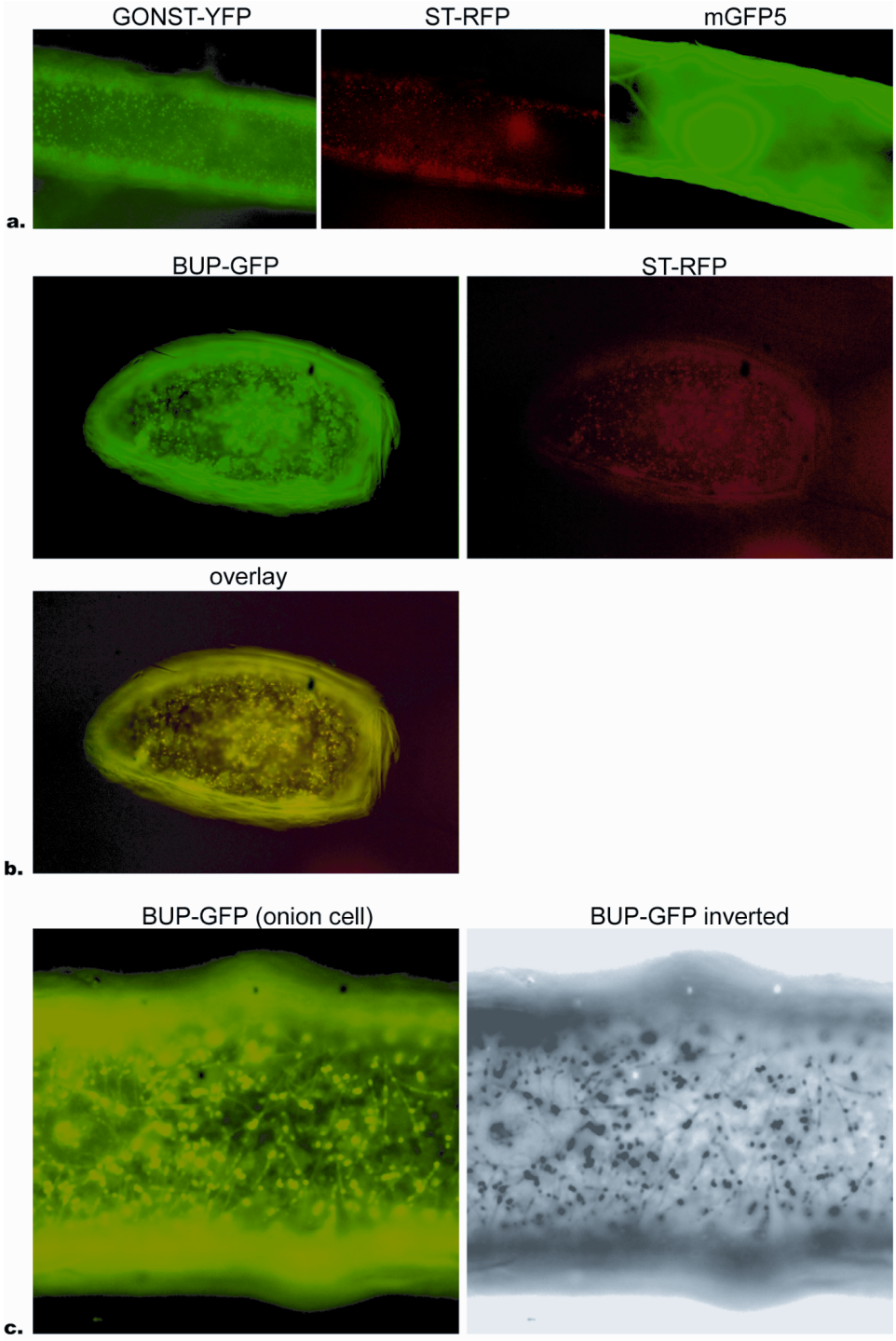
Chapter 5 and 6

Chapter 5, figure 2: GUS histochemical localization of BUP expression



GUS staining of *BUP::GUS:GFP, 35S::GUS:GFP* and *LAT52::GUS:GFP* transformants showed similar expression patterns. As an exception, the pollen of *LAT52::GUS:GFP* transformants were also stained for GUS.

Chapter 6, figure 2: BUP-GFP protein fusion localization in onion and *Arabidopsis* cells



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Chapter 6, Figure 2: BUP-GFP protein fusion localization in onion and *Arabidopsis* cells
Transiently (onion) and stably (*Arabidopsis*) transformed cells were analyzed for localization of the BUP-GFP fusion protein, using wide field fluorescence microscopy. a. Golgi-markers GONST-YFP and ST-RFP, and the cytoplasmic localization of the soluble mGFP5 of the pCAMBIA 1302 construct in onion epidermal cells, b. Localization of BUP-GFP (green) and ST-RFP (red) in onion epidermal cells. Co-localization (yellow) is shown in the overlay c. BUP-GFP localization in fibre-like strands in onion cells, d. BUP-GFP localization in *Arabidopsis* epidermal root cells